BINDING MECHANISM OF K88AB PILI PRODUCED BY ENTEROTOXIGENIC *Escherichia coli*

by

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LIST OF ABBREVIATIONS

AMP	Adenosine monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphophate
CFA	Colonization factor antigen
EDTA	Ethylendiaminetetraacetic acid, disodium salt
EGTA	Ethyleneglycol-bis-(β-aminoethylether) N,N,N',N'-tetraacetic acid
ETEC	Enterotoxigenic *Escherichia coli*
GMP	Guanosine monophosphate
LT	Heat-labile enterotoxin
PBS	Phosphate-buffered saline
PMSF	Phenylmethylsulfonyl fluoride
SDS	Sodium dodecyl sulfate
ST	Heat-stable enterotoxin

CHAPTER ONE

INTRODUCTION

Enterotoxigenic Escherichia coli (ETEC) is one of the most important causative agents of diarrheal disease in human infants in developing countries and in young animals (63, 158). The mechanism of pathogenesis by ETEC includes adherence to epithelial mucosa by means of adhesive pili on the bacterial surface, colonization of small intestine, and subsequent secretion of enterotoxins (58). K88 pili are produced by ETEC strains commonly associated with outbreak of diarrhea in piglets (91, 180). K88 pili are further classified into three major subtypes, K88ab, K88ac, and K88ad based on their immunological cross-reactivity (71).

Studies comparing binding of K88 by intact and periodate-oxidized brush border membrane and mucus and inhibition of binding by amino sugars, glycopeptides, and glycoproteins indicated that receptors for K88-positive ETEC and purified K88 pili are glycoconjugates with carbohydrate as an essential recognition site (8, 65, 107, 153). However, the actual carbohydrate structures which are responsible for binding of K88 have not yet been clearly determined. Terminal β-D-galactose (65), galactose (153), and terminal N-acetylglucosamine and N-acetylgalactosamine (8) have all been reported to be involved in binding of K88-positive ETEC to small

intestinal epithelial cells.

Milk and colostrum are known to contain immunoglobulin, lysozyme, lactoferrin, and lactoperoxidase which have antibacterial properties (99, 141, 142, 145). Immunoglobulins provide protection to neonates through passive immunity (16, 17) and by preventing adhesion of enteropathogens to the intestinal mucosa (92, 127, 154). Vaccination of sows with pili from enterotoxigenic Escherichia coli (ETEC) prior to farrowing protects neonatal piglets from diarrhea (146). However, milk and colostrum may also be abundant sources of non-immunoglobulin substances which inhibit adhesion of ETEC to intestinal mucosa (142). Binding of K88ab pili involves recognition of specific receptor structures on brush border membranes. Non-immunoglobulin substances in milk and colostrum may have structures similar to these receptors. For example, glycopeptide (65) and milk fat globule membrane (MFGM) (142) from sow milk have been shown to inhibit hemagglutination mediated by K88-positive strains. MFGM, which originates from apical plasma membrane of mammary epithelial cells (46, 116), may possess receptors similar to intestinal brush border membrane. Electron microsopy has been used to demonstrate attachment of K88-positive E. coli to milk fat globule membrane (142). Moreover, K88-positive strains attach to MFGM prepared from

sow milk but not from cow milk (142). Other studies have shown that human milk contains various non-immunoglobulin fractions which may inhibit heat-labile enterotoxin (LT) produced by ETEC and cholera toxin (80, 81, 133), as well as prevent adhesion of V. cholerae (hemagglutination) (80, 81). Gangliosides from human milk inhibit fluid secretion induced by cholera toxin and ETEC LT in the rabbit intesinal loop assay (133).

The objectives of the present study were (1) to identify and characterize the receptor for K88ab pili in pig brush border membrane and mucus, and (2) to isolate and identify milk and colostrum components which inhibit binding of K88ab pili to intestinal brush border membrane.

CHAPTER TWO

REVIEW OF LITERATURE

A. Structure and Function of Brush Border Membrane

Brush border membrane is a trilaminar membrane, which covers the microvilli of columnar epithelial cells in the small intestine. Glycocalyx, which originates from brush border membrane glycoprotein, appears in the transmission electron microscope as a layer of fine irregular filaments lining the microvilli surface. Controlled proteolytic digestion, in vitro, readily removes the glycocalyx from brush border membrane (53, 90). The brush border membrane is rich in protein and glycoprotein and the high protein to lipid ratio (1.7:1) (54) has been correlated with the large number of intramembrane particles observed in the transmission electron microsopy (125). Brush border membrane proteins have been resolved into 20 distinct electrophoretic bands with apparent molecular weights ranging from 17,000 to 380,000 (5, 139). Receptor proteins that selectively bind vitamin B_{12} (113) and Ca^{2+} (102), as well as transport proteins that actively absorb D-glucose and amino acids (30), are present. Moreover, a number of hydrolytic enzymes, including various disaccharidases and peptidases, are distributed along the brush border membrane (30, 26, 35). These enzymes contain a hydrophilic domain, which is exposed to the lumen; a hydrophobic anchor; and a

trans-membrane domain, which is accesible from the cytoplasmic surface. The hydrophilic domain, which contains the enzymatic active site and is glycosylated, contributes more than 95 % of the mass (96).

The lipid component of brush border membrane is unusually rich in cholesterol and glycolipid (34, 62). The functional significance of cholesterol and glycolipid enrichment is unclear. Both the high protein to lipid ratio and the high cholesterol and glycolipid content appear to contribute to low membrane fluidity (99). The only known function attributed to any glycolipid component is the selective binding of cholera toxin and LT by the ganglioside G_{M1} (47, 50, 85).

B. Structure and Function of Gastrointestinal Mucus

Gastrointestinal mucus is a slimy and visco-elastic secretion which covers the epithelial surfaces of the small intestine. The major function of mucus is to lubricate the intestinal wall and to protect the mucosal tissue from physical and chemical injury from food solid, digestive fluid, and/or bacteria. Mucus consists of glycoprotein which is responsible for the viscous and gel-forming properties. The purified glycoprotein from mucus, which is called mucus glycoprotein or mucin, has a molecular weight greater than 10^7 and contains high amounts (usually more than 50 % of mass) of carbohydrate.

Mucus scraped from the intestinal wall contains free protein, nucleic acid, and lipid, in addition to mucus glycoprotein. The high viscosity of mucus hampers isolation of mucus glycoproteins from non-covalently bound proteins and nucleic acid. Thiol-containing reagents, sonication, and chaotropic reagents have been used to reduce the viscosity of the mucus and proteolytic enzymes have been used to degrade free protein present in the secretion. However, mucus glycoprotein prepared using these procedures also undergoes a chemical and physical changes (31). Mild separation methods, such as sol-gel separation, homogenization, chromatography, and density gradient ultracentrifugation, have been used satisfactorily. Actual selection of methodology depends upon the properties of mucus gel and final use intended for the preparation (31). Equilibrium ultracentrifugation in a CsCl gradient, followed by gel filtration on Sepharose 4B, has been used successfully to purify small amounts of gastrointestinal mucus glycoprotein, which retains the characteristic visco-elastic properties (114).

Pig small intestinal mucus glycoprotein has a molecular weight of 1.7×10^6 (115). The polymeric structure can be disrupted by reduction with 0.2 M mercaptoethanol into glycoprotein subunits (2.4×10^5) (115). Addtionally, a 90,000 molecular weight protein,

called link peptide, is released upon reduction on a 1:1 molar basis (by weight) (115). Pig and human gastric mucus glycoproteins have a structure simpler than pig small intestinal mucus glycoprotein. Pig gastric mucus glycoprotein has a molecular weight of 2×10^6 and consists of four glycoprotein subunits apparently joined by disulfide bond with a central 70,000 molecular weight protein (link peptide) (161, 135, 3). The link protein contains high amounts of cystein but no carbohydrate (135). Both human gastric mucus glycoprotein and subunits have similar molecular weight as their pig counterparts (136), but the detailed structures are unknown.

Two models have been proposed for the polymeric structure of mucus glycoprotein. In the windmill model proposed by Allen and his groups (4, 161), the non-glycosylated link peptides in the center of polymeric mucin link mucin monomers together. The arrangement of subunits is similar to vanes of a windmill around their axis (4). In the flexible thread model, Carstedt et al. (22, 23) suggested that mucus glycoprotein subunits are attached end to end by disulfide bonds. They proposed that link peptides are originally part of subunits, each of which contains non-glycosylated and glycosylated regions. Proteolytic enzymes present during extraction `nick' the non-glycosylated region and produce peptides which appear

after thiol reduction. Mucus glycoproteins are distinguished by their microheterogeneity, also referred to as polydispersity (64), with respect to size, density, and charge. Thus, mucins are not homogenous, even when isolated from an individual animal or a single organ. The microheterogeneity reflects variations in the composition of oligosaccharide chains (178).

In mucus glycoprotein, numerous oligosaccharide chains are linked to the peptide core by O-glycosidic bonds between N-acetyl galactosamine residues and serine and/or threonine (56, 101). The oligosaccharides contain N-acetyl- or N-glycolylneuraminic acid, fucose, galactose, N-acetylglucosamine, and N-acetylgalactosamine. Trace quantities of mannose and glucose have been reported for purified gastrointestinal mucus glycoproteins. Sulfate ester residues also occur in an internal position within the oligosaccharide chains (156). The peptide core is composed primarily (over 70 %) of serine, threonine, and proline. The glycosylated regions of the peptide, which are rich in these amino acids, are resistant to proteolysis.

The size of carbohydrate side chains and the relative amounts of the different sugars vary between the different gastrointestinal mucins. Side chains range from 2 to 15 sugars in length and some are branched. Carbohydrate side

chains can be considered to have three structurally distinct domains, namely, a core region incorporating protein link; a backbone region, which may be linear or branched; and a peripheral region. Each region expresses various antigens, which are related to blood group antigens (104). Pig gastrointestinal mucin expresses A and O blood groups (114).

The most prominent physical characteristics of mucin are the high viscosity as well as ability to form gels. Pig small intestinal mucus glycoprotein possesses a high intrinsic viscosity (500 ml/g). As the concentration of glycoprotein increases from 9 mg/ml to 10-12 mg/ml, the viscosity increases asymptotically until gel formation occurs (114). The concentration of glycoprotein occurring in native mucus gel on the small intestinal surface is 10 mg/ml. Mucus gel is sufficiently strong to resist the force of solubilization. However, mild shear can break the gel.

The ability of mucus to form gels has been attributed to the polymeric structure of the constituent glycoproteins (3, 161). Both reduction and proteolysis of mucus glycoprotein results in solubilization of the gel. Reduced and degraded glycoprotein subunits have much lower viscosities than native mucus glycoprotein. The effect of carbohydrate side chains on gel formation is not yet known.

The high carbohydrate content ensures that mucus glycoprotein occupies a large solution volume and may contribute to molecular interaction of the gel matrix (56).

The most important function of mucin may be protection of epithelial cells from the physical and chemical damage that can occur during digestion. Mucus provides lubrication for passage of ingested foods through the gut with a minimal of shear force (52). Mucus protects gastric and duodenal mucosa from excesses of acid in the lumen by retaining bicarbonate secreted by mucosa (75). Since hydrogen ion can permeate the gel, a pH gradient is established across the mucus layer (179). Although proteolytic enzymes cannot penetrate the mucus layer, degradation of mucus does occur at the luminal surface (2). In order to maintain the integrity of the layer, mucus must be continuously secreted. Mucus may also form a physical and chemical barrier against bacterial colonization of intestinal mucosa. The high viscosity of mucus retards the mobility of bacteria and inhibits access to epithelial cells. Whether adhesion of bacteria to mucus facilitates colonization of intestines is presently unknown (8. 9, 52, 150). Adhesion to mucus may be either an initial step in adherence to epithelial cells and colonization of the intestinal tract or result in elimination from the intestinal tract as mucus is released into the lumen.

C. Enterotoxin Production by ETEC

ETEC are able to reach the small intestine of newborn piglets through the stomach, which still has a pH near neutrality. Thus far, all strains of ETEC reported to produce diarrhea are able to overcome peristalsis and colonize the small intestine by adherence to the brush border memberane. Enterotoxins, which are secreted very near their receptor sites, induce the secretion of water and electrolyte into the lumen, resulting in diarrhea, dehydration and death (72). Two types of enterotoxins can be distinguished on the basis of their theromostability. One is designated heat labile toxin (LT) and the other heat stable toxin (ST).

LT is a large molecular weight protein, which has been categorized as LT-I or LT-II based on animal origin and by antigenic specificity against cholera enterotoxin (CT) (69, 78, 138). LT-I consists of one 28,000 Mr subunit (A) and five 11,500 Mr subunits (B) (26, 103, 62). B subunit contains 103 amino acid residues (138, 69). LT-I has been isolated from strains causing diarrhea in humans and pigs. LT-1 possesses antigenic determinants both common and unique to CT and can be neutralized by antitoxin against CT (12, 25, 27). Biological activity of LT-I has been ahown to be similar to choleragen in several different assay systems. LT-I from human and porcine ETEC possess unique

antigenic determinants which reside exclusively in the B subunits (62, 84, 173) and are manifest by differences in the sequence of signal peptides as well as amino-terminal ends (182, 183).

The toxic function of LT-I resides in the A subunit, which activates adenylate cyclase by catalyzing a covalent ADP ribosylation of the regulatory subunit of that enzyme. Adenylate cyclase catalyzes decomposition of adenosine triphosphate (ATP) to cyclic 3', 5'- adenosine monophosphate (cyclic-AMP) and pyrophosphate (49, 66). Increased intracellular levels of cyclic AMP results in active secretion of chloride ions into the intestinal lumen and subsequent loss of fluid characteristic of diarrhea. The B subunits of both LT-I and cholera toxin are believed to bind with host cell membrane receptors containing ganglioside G_{M1} (40, 79, 83, 124).

LT-II is produced by ETEC isolated from a water buffalo in Thailand (138). Although LT-II resembles LT-I with respect to biological activity, LT-II is not neutralized by antiserum aganist CT or LT-I. LT-II consists of two different polypeptides with Mrs of 28,000 (A) and 11,800 (B). The activity of LT-II is not blocked by ganglioside GM1 (77).

The two heat stable toxins (STa and STb) are encoded on different genes (162) and are differentiated by methanol

solubility and biological activity. STa, which is produced by porcine, human and bovine strains of ETEC, is active in suckling mice and piglets. STa specifically activates the particulate fraction of guanylate cyclase in intestinal mucosal cells resulting in increased cellular cyclic GMP levels and a net increase in intestinal fluid secretion (51, 140). STa is soluble in methanol and shares similar antigenic determinants when prepared from different strains (140). Two types of STa have been reported that differ in amino-terminal sequences; one contains 18 and the other 19 amino acid residues (1, 108, 148, 172). STa contains 6 half cysteine residues per molecule and is devoid of basic amino acids. Biological activity can be inhibited by treatment with 0.1 M 2-mercaptoethanol or dithiothreitol suggesting that disulfide bonds are essential (167). STb has been identified only in porcine ETEC strains and is methanol insoluble. STb is biologically active in piglets, but not in suckling mice. Although presently unknown, the mechanism of action does not involve increases in intestinal mucosal cyclic GMP (140). DNA sequence of the gene encoding for STb indicates the presence of 71 amino acid residues (137). Human ETEC strains which hybridize with a DNA probe for genes encoding for STb were found in human specimens from Thailand (44) but not from Brazil and Bangladesh (176). In contrast to pig jejunum, human ileal

tissue does not respond electrogenically to STb (176).

D. Pili-Mediated Adhesion of ETEC

Adhesion of ETEC to enterocyte is necessary for colonization of small intestine. Adhesion is mediated by pili, which appear as nonflagellar, filamentous structures on the bacterial cell surface and which consist of numerous polypeptide subunits. Pili are thinner and more numerous than flagella and confer adhesive properties on the bacteria as well as hemagglutinating ability (58).

Type 1 fimbriae are the most common type of pili present on the surface of both pathogenic and nonpathogenic E. coli strains. These fimbriae enable E. coli to adhere to a wide variety of eukaryotic cells (42). The adhesive properties of type 1 fimbriae are inhibited by D-mannose and oligomannoside-type glycopeptide from ovalbumin (131). These fimbriae can aid colonization of epithelial surfaces through their adhesive properties and, therefore, contribute to the pathogenicity of enteropathogenic and enterotoxigenic E. coli (42, 89).

Pili produced by ETEC are host-specific, i.e. adhere to intestinal epithelia of a very limited number of animal species and specifically agglutinate erythrocytes obtained only from certain species. In contrast to type 1 fimbriae, the hemagglutinating abilities of these pili are not inhibited by mannose (58).

Outbreaks of porcine neonatal diarrhea are commonly associated with ETEC which bear the K88 pili on their surface. ETEC strains that produce diarrhea in pigs, but lack the K88 pili, often possess 987P pili (128). ETEC strains isolated from calves and lambs with enteritis possess K99 and/or F41 pili (132). Additionally, F41 pili have recently been reported to be associated with ETEC strains which cause diarrhea in neonatal piglets (159, 39). CFA/I and CFA/II are immunologically distinct surface antigens present on human ETEC (47, 48).

E. Pili Characteristics

1. K88 Pili

K88 pili vary in length from 0.2 to 1.3 μm and in diameter from 80 to 130 A (168). Pili form through aggregation of subunits which have molecular weights from 23,500 to 26,000 depending on the K88 variants from which they are obtained (118). All K88 variants, designated K88ab, K88ac, and K88ad, possess a common antigenic determinant, K88a. Adhesiveness appears to be associated with K88b or K88c antigens but not K88a (97, 180). K88ab has been further differentiated into K88ab1 and K88ab2, which were isolated from different strains (43). All K88 variants have similar amino acid compositions and are devoid of cysteine residues (118).

Nucleotide sequences of the deoxyribonucleic acid of

the genes encoding K88ab1, K88ab2, K88ac and K88ad pili (43, 59, 60, 61) as well as the amino acid sequence of K88ab pili (60, 97) have been determined. K88ab pili are synthesized in a precursor form with an additional 21 amino acid residues located at the N-terminal end (60, 97). The N- and C-terminal ends, which are identical in all four K88 variants (61, 43), appear to be involved in stabilization of the polymeric structure while the central portion contains the region which determines specific antigenicity. Although K88 antigen is considered to exist as a nascent polypeptide chain (117), K88ab contains sites in the primary structure with potential for N-glycosidation (58).

K88-positive strains agglutinate guinea pig erythrocytes in the presence of mannose at 0-3 C (91). Hemagglutination does not occur with K88-negative mutants or when K88-positive strains are grown at 18 C. Parry and Porter (134) reported that K88ab strains also adhere to chicken erythrocyte membrane but that K88ac strains do not. Adhesion of K88ab strains to chicken erythrocytes is stable at room temperature and is mannose resistant. Parry and Porter (134) suggested that similarities exist between the ability of K88 to hemagglutinate certain erythrocytes and to mediate attachment to the intestinal mucosa of piglets (134).

K88 pili occur on strains of ETEC with serogroup O8,

O45, O138, O141, O147, O149, and O157 (163, 165). The presence of K88 is associated with strains which produce ST only, LT only, or both enterotoxins (58).

2. K99 Pili

K99 pili were first purified from E. coli K12 harboring a K99 plasmid. These pili consist of individual subunits which aggregate to form rod-like filamentous structures with an average diameter of 8.4 nm and a mean length of 130 nm (85, 87). K99 has a monomer molecular weight of 21,300 based on amino acid composition and a pI of 10.1 (84). However, when K99 pili were purified from E. coli K12 W3110 strains (harboring a K99 plasmid which originated from E. coli B41), the purified monomers had a molecular weight of 18,500 and pI of 9.5 (38). This preparation contained a predominance of apolar amino acids and cysteine and the N-terminal amino acid sequence was not homologous with K88. K99 antigen was also purified from E. coli strains obtained during outbreaks of calf scours (6, 7). The monomer molecular weight attained for this preparation was only 13,000 and the N-terminal amino acid was blocked. Cysteine and methionine were absent from this preparation.

Differences reported in molecular weight and pI for various preparations of K99 in molecular weight and pI were also reflected in the hemagglutination patterns obtained.

K99 prepared from E. coli B41 (38) hemagglutinated sheep and horse erythrocytes, while K99 purified from wild strains (7) hemagglutinated horse, but not sheep, erythrocytes in the presence of mannose. K99 pili appear to occur rather commonly among porcine strains of ETEC typing O64 and O101 and occasionally among O9 strains (58). In vitro production of K99 is repressed by alanine (37, 58) and by the inhibitory products which form by Maillard's reaction in glucose-rich media during autoclaving (58). Minimal salt medium with glucose (38) and semisynthetic medium (37) are more suitable for K99 antigen production than commercially available complex media such as nutrient broth. In some strains, K99 production is constitutive and independent of the presence of glucose; in other strains, K99 production is glucose dependent (67, 68). The production of K99 and F41 pili occurs during balanced growth with a high biomass yield in logarithmic phase, but not in stationary phase. The optimum pH of medium for the production of these pili is 7 (175).

3. F41 Pili

F41 was first partially purified from E. coli B41, which also produces K99 pili, and mistakenly identified as K99 (121). The isolated pili had a pI of 4.2 and hemagglutinated guinea pig, as well as sheep, erythrocytes in the presence of mannose. Subsequent studies (87, 122,

123) concluded that E. coli B41 produces two antigenically distinct pili: a cationic (K99) and an anionic (F41) component. F41 antigen has been purified from a mutant of E. coli B41 which does not produce K99 pili and has a monomer molecular weight of 29,500. The F41 pili have diameters of 3.2 nm and are slightly thinner than K99 pili. The N-terminal amino acid sequence of F41 shows limited homology with K99 (39).

F41 antigen has strong hemagglutinating activity with guinea pig and human group A erythrocytes and weaker hemagglutinating activity with horse and sheep erythrocytes. The production of F41 pili is repressed by alanine (39). F41 pili are associated with K99 positive strains of serogroups O9 and O101 (58).

4. 987P Pili

987P pili have diameters of 7 nm and axial holes and are composed of protein subunits with an apparent molecular weight of 18,000 (19). 987P has a pI of 3.7 and contains an unidentified amino sugar (88). 987P-positive ETEC consistently yield a greater proportion of pilated cells when obtained from pig small intestine than when grown in vitro. Strains containing 987P pili can adhere to intestinal epithelium in vivo (128).

F. Specificity of ETEC Adhesion

The adhesion of ETEC producing K88, K99, F41, 987P,

CFA I, and CFA II is host-specific. For example, strains isolated from pigs with diarrhea fail to elicit a response in calves and lambs after oral challenge. Failure to produce diarrhea results from inability of ETEC to adhere to and proliferate in the anterior small intestine of calves and lambs. However, enterotoxigenic strains isolated from calves or lambs with diarrhea produce severe clinical symptoms when administered orally less than 20 hours post-partum to colostrum-fed calves and lambs (158). Although K88-positive E. coli also adhere to human duodenal cells (36), ETEC strains producing K88, K99, F41, and 987P pili are significantly less adhesive to human ileal enterocytes than CFA-positive ETEC (98).

Individual piglets vary in susceptibility to ETEC at different ages as well as in localization of ETEC colonization within the small intestine. K88-postive strains have been isolated from diseased pigs during both the neonatal and post-weaning periods (165). Strains possessing this pili adhere primarily to the anterior portion of the small intestine. However, K99-positive strains adhere to the middle and posterior portions of the small intestine (87, 158) and produce diarrhea primarily in neonatal piglets. No reports have appeared linking K99-positive strains to production of diarrhea in pigs after weaning (119, 159). Likewise, calves and lambs

become resistant to experimental challenge with K99-positive strains after 2 days of age. The intestinal epithelium of pigs and calves appears to become resistant to K99-mediated adhesion with increasing host age (144). Strains possessing the 987P pili adhere primarily to the posterior small intestine of piglets (144, 129).

Some piglets appear to be genetically resistant to infection with K88-positive ETEC strains. Initially, the existence of at least two porcine phenotypes was described (152). Adhesion-positive piglets are susceptible to infections with K88-positive ETEC strains while adhesion-negative piglets are resistant. Piglet phenotypes have been screened according to ability of brush border preparations to bind K88ab, K88ac, and K88ad antigens (13, 14). At least five different porcine phenotypes have been distinguished. Pigs of one phenotype were susceptible to adherence by all there variants, pigs of three phenotypes were susceptible to only one or two of the K88 variants and pigs of one phenotype were resistant to adhesion by all variants.

G. Mechanisms of ETEC Adhesion

Both bacterial and animal membranes possess an overall net negative charge. In order to attach to animal cell surfaces bacteria must overcome the charge repulsion which exists. At relatively long distances (> 10 nm) between

planar bodies, the attractive forces exceed the repulsive forces (147). At this distance, forces are easily reversed by fluid shear. However at shorter distances, the repulsive force increases relative to attractive force. Bacteria are unable to overcome these repulsive forces and approach animal cells. However, structures with small radii can approach closely (1 nm) where attractive forces are stronger. The filamentous structures of pili, which have radii from 1 to 5 nm, provide a bridge between bacteria and epithelial cells without encountering repulsion (147). Hydrophobic interaction may also be involved in adhesion of pili to animal membrane surfaces (160). Piliated E. coli have greater hydrophobicity than nonpiliated E. coli. Hydrophobic areas located in the binding site may be involved in adhesion of pili to brush border membrane. Host specificity and age dependence would suggest that specific binding of pili by receptors on the surface of intestinal epithelial cells is involved in adhesion of ETEC to intestinal mucosa (11).

Loss of receptor activity following treatment of brush border membrane and mucus with sodium metaperiodate suggests that most receptors of ETEC pili are glycoconjugates with carbohydrate as an essential recognition site (65, 107, 153). However, the biochemical natures of K88 pili-specific receptors have not yet been

clearly elucidiated. Inhibition experiments (153) involving binding of ^{125}I-labeled K88 pili to porcine brush border after addition of simple mono- and oligosaccharides suggests that galactosyl residues on the intestinal brush border membrane are involved in binding of K88 pili. Binding was reduced by addition of stachyose, galactan, amino sugars and compounds containing free amino groups. Treatment of brush border membrane with formaldehyde makes possible differentiaton between adhesive and non-adhesive brush border membrane, while treatment with either periodate or glutaraldehyde decreases receptor activity (153). Studies based on the ability of mucus glycoproteins to inhibit agglutination of guinea pig erythrocytes by K88 antigen suggested involvement of terminal β-galactosyl residue in the agglutinating ability of K88 (65). However, inhibitory activity could not be located in either the peptide fraction or the carbohydrate fraction of porcine submaxillary glycoprotein after alkaline degradation in the presence of borohydride. Inhibition studies using glycoproteins and mucins suggested that both terminal N-acetylglucosamine and N-acetylgalactosamine were involved in adhesion of K88 antigen to porcine brush border (8). Adhesion of $^{35}SO_4$-labeled E. coli producing K88 pili to mouse small intestine mucosa was decreased by pretreatment of mucosa with trypsin, pronase, or sodium metaperiodate as

well as by the addition of D-galactosamine (107). Two receptor proteins, with molecular weight of 57K and 64K, and three receptor proteins, with molecular weight of 57K, 64K, and 91K were identified in mucus and intestinal brush border, respectively (107). Brush border fractions isolated from adhesion-positive and adhesion-negative pigs differed only in their glycolipid profiles. Thus, glycolipids present in brush border membranes from adhesion-positive pigs were suggested to be receptors for K88 pili (94).

Addition of mono- and disialogangliosides, especially G_{M2} at concentrations above the critical micellar concentration, inhibits hemagglutination of sheep erythrocytes by K99-positive strains (50). Pretreatment of erythrocytes with trypsin and pronase has no effect on hemagglutination and suggests that glycolipids may be involved in the receptor for K99 pili on sheep erythrocytes. However, in another study using human, porcine, and equine erythrocytes, the receptor for K99 appeared to be a glycophorin possessing a NANA α 2-6 GalNAc structure (109). The receptor for K99 pili was isolated from equine erythrocytes and characterized as equine erythrocyte hematoside (157).

ETEC producing 987P pili adhere in vitro to small intestinal villous epithelial cells and brush border

isolated from adult female rabbits, but not from infant rabbits (33). Receptors for 987P were localized by immunocytochemistry along the entire villous surface and in goblet cells in adults. However, in infant rabbits receptors could only be detected in goblet cells (34). Purified 987P receptors from adult rabbits are low-molecular-weight glycoproteins (<14,000) with isoelectric points are of 2.2, 3.8, and 4.1. The 987P receptor activity is sensitive to periodate oxidation and to digestion with pronase (35). Receptor activity is inhibited by amino sugars and their N-acetylated derivatives and by compounds possessing free amino groups (35).

H. Inhibitory Substances in Milk and Colostrum

Piglets acquire passive immunity from the sow through ingestion of colostrum (16). The major immunoglobulin present in sow colostrum is IgG, which is derived directly from blood through uptake by the mammary gland (17). Since much of the immunoglobulin present in colostrum is absorbed intact by the neonatal piglet, the selective secretion of serum IgG into colostrum provides a mechanism for transfer of serum antibody from the dam into the circulation of the neonate (17). When gut closure occurs, intact colostral proteins are no longer absorbed from the intestine. During the transition from colostrum to milk, the IgG

concentration falls dramatically and IgA becomes the major immunoglobulin class in milk. However, the absolute concentration of IgA in milk also falls in comparision to colostrum. IgA and IgM in milk are synthesized almost entirely within the mammary gland (20). IgA in milk can prevent enteric disease in suckling piglets by localized action within the intestinal lumen (117). Milk secreted by sows immunized with inactive E. coli can protect piglets against diarrhea caused by ETEC (99). Antibody in milk and colostrum may either suppress the multiplication of E. coli in the small intestine (99) or kill bacteria in combination with lactoferrin (20). However, Rutter and Anderson (146) could find no correlation between the antibacterial activity in colostrum and milk and protection against enteric disease in piglets.

Antibodies in milk and colostrum can also prevent adhesion of ETEC to the intestinal mucosa. Sows immunized with purified K88 pili provide protection from experimental challenge with K88-positive ETEC to their offspring (145). Colostrum and milk from immunized sows were shown in vitro to possess anti-adhesive activity. This anti-adhesive effect has been shown to correlate in vivo with protection of the suckled pig (127). Colostrum from adhesion-positive sows inhibited binding of purified K88 antigen to brush borders significantly better than colostrum from

adhesion-negative sows. Vaccination of sows with 987P or K99 pili also protects neonatal pigs against diarrheal disease caused by ETEC strains possessing these antigens under controlled experimental conditions. This protection did not extend to enterotoxigenic strains possessing other adhesins (86, 120).

Carbohydrate compounds which inhibit pili-mediated bacterial adhesion as well as toxin binding to intestinal receptors have also been identified in colostrum and milk. Human milk contains a nonimmunoglobulin fraction which is more inhibitory toward bacterial adhesion, hemagglutination and binding of enterotoxin produced by E. coli and V. cholerae than the immunoglobulin fraction (79). Apparently components present in human milk and colostrum serve as analogs of brush border receptors for some bacterial adhesins and enterotoxins. In a subsequent study (81), glycoprotein and oligosaccharide present in human milk whey were shown to inhibit classical V. cholerae hemmaglutination while a high-molecular-weight glycoprotein inhibited El Tor V. cholerae hemagglutination. In each case, carbohydrate appeared to be involved, since the inhibitory activity resisted boiling, was destroyed by periodate treatment, and was bound by concanavalin A-Sepharose. Sow colostrum also contains components which inhibit hemagglutination of guinea pig erythrocytes by K88.

Inhibitory activity was detected both in a low molecular weight glycopeptide from colostrum (65) and in milk fat globule membrane from milk (141). K88-positive E. coli attach to milk fat globule prepared from milks of adhesion-positive sows but not when prepared from milks of adhesion-negative sows (9). Milk fat globule membrane also inhibits hemagglutination by K99-positive E. coli (141).

Gangliosides from human milk inhibit fluid secretion induced by cholera toxin as well as by ETEC LT in the rabbit intestinal loop assay (133). A component with mobility similar to G_{M1} in thin layer chromatography was associated with this inhibitory activity. A ganglioside fraction, which completely inhibits 0.1 μg of cholera toxin in rabbit intestinal loop assay, is present in trace amounts in human milk.

Previous studies indicate that K88 binding is inhibited by treatment with periodate and proteolytic enzyme. Although glycoproteins have been suggested as receptors for K88 pili in brush border membrane and mucus, the specific carbohydrate structure recognized has not been clearly elucidated. In the present study the mechanisms of K88 binding by brush border membrane and mucus were determined by using Western blot and inhibition assays. Neonatal piglets are more susceptible to diarrhea caused by ETEC than are adult hogs. This age-dependence of ETEC

enteritis could be correlated with decreases in receptor for K88 pili in the brush border membrane and mucus of older pigs. In the present study, changes which occur in polypeptide patterns and K88ab receptor patterns of brush border membrane and mucus prepared from pigs at different ages were determined. Previous studies indicate that sow colostrum and milk contain substances which inhibit the K88-mediated hemagglutination of guinea pig erythrocytes. In the present study fractions of sow colostrum and milk, as well as cow milk, which possess the ability to inhibit the binding of K88ab pili by brush border membranes were identified. Milk proteins which possess the ability to bind K88ab pili will be further identified by Western blot assay.

CHAPTER THREE

MATERIALS AND METHODS

A. Materials

Molecular weight markers (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, α-lactalbumin, thyroglobulin, ferritin, catalase, lactate dehydrogenase), Sepharose CL-4B, Sepharose 4B, and Con-A Sepharose were purchased from Pharmacia Fine Chemicals, Piscataway, New Jersey. Dialysis tubings were purchased from Spectrum Medical Industries, Inc., Los Angeles, California. Coomassie brilliant blue R, trypsin type XI, pronase, ethyleneglycol-bis-(β-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA), ethylendiaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), concanavalin A tris(hydroxymethyl)-aminomethane (Tris), 2-mercaptoethanol, mannitol, chloramine-T, galactosamine, glucosamine, mannosamine, N-acetylglucosamine, N-acetylgalactosamine,N-acetylneuraminic acid, α-methylmannose, β-methylgalactose, α-methyl-galactose, β-methyl-N-acetylglucosamine, β-methyl-N-acetylglucosamine, and immunochemicals were from Sigma Chemical Co., St. Louis, Missouri. Anti-rabbit IgG-horseradish peroxidase, o-dianisidine, and Tween-20 were purchased from Bio-Rad, Richmond, California. Trypticase soy broth was from Baltimore Biological

Laboratories, Cockeville, Maryland. 125-Iodine carrier-free, sodium iodide with specific activity of 15.1 mCi ^{125}I/μg was purchased from Amersham, Arlington Heights, Illinois. All other chemicals were of reagent grade quality. All concentrations denoted by percentage are weight-volume unless otherwise specified.

A strain of ETEC with serotype O8:K87:K88ab:H19 was obtained from the E. coli Reference Center at Pennsylvania State University, State College, Pennsylvania. Anti-K88ab serum was prepared by immunizing a rabbit with the isolated K88ab pili. K88ac pili and anti-K88ac serum were provided by A. W. Way at Department of Food science and Technology. laboratory.

B. Preparation of Brush Border Membrane

Brush border membrane was prepared by a modified procedure (18) using 2-, 21-, and 42-day-old piglets and hogs obtained at the University Swine Center and the University Abattoir, respectively. Piglets were sedated with chloroform and euthanized by an intracardial injection of sodium pentobarbital (0.5 ml/kg body weight). Ventral surface was immediately washed with ethanol and abdominal cavity was opened using surgical instruments. The small intestine was removed, cut into several parts of appropriate length and flushed with ice-cold 0.15 M NaCl. The sections were then clamped and filled with buffer A

(96 mM NaCl, 8 mM KH_2PO_4, 5.6 mM Na_2PO_4, pH 6.8, 1.5 mM KCl, and 10 mM EDTA, pH 6.9), the other end was clamped and the intestines were immersed in ice-cold 0.3 M sucrose for 30 min. Intestines were gently rubbed along their length with fingers and the resulting cell suspension collected. Intestine sections were refilled with buffer A and the procedure was repeated. Cell suspensions were combined and cells harvested by centrifugation (Sorvall RC-5B centrifuge) at 1,000 x g for 10 min at 4 C. Cells were resuspended in buffer B (4 mM EDTA, 1mM EGTA, 10 mM imidazole, pH 7.3) and were disrupted in a Waring blender at half of a full speed for 30-40 sec. After centrifugation of homogenates at 3,300 x g for 10 min at 4 C, pellets were washed twice with buffer B and once with buffer C (75 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM imidazole, pH 7.3). After suspension in a small volume of buffer C, preparations were carefully layered on a sucrose step gradient and centrifuged at 100,000 x g (Beckman ultracentrifuge) for 60 min at 4 C. The gradient contained 2 ml of 65 % sucrose, 4 ml of 50 % sucrose and 4 ml of 25 % sucrose in buffer C. Enriched preparations of brush border membrane were obtained by collecting material which banded at the 25/50 % interface. Preparations were washed once with buffer C, collected by centrifugation at 10,000 x g for 15 min at 4 C and stored at - 20 C until further use.

C. Preparation of Small Intestinal Mucin

Small intestinal mucin was prepared by a modified procedure of Robertson and Stanley (143). Mucin was collected from 2-, 21-, and 42-day-old piglets and hogs. The jejuno-ileum was excised from the small intestine and used for mucin preparation. Digestive material was removed by gently flushing intestine with ice-cold physiological saline (0.15 M NaCl). Mucus gel was collected by scraping the mucosal surface with a glass slide and was dispersed in 2 volumes (v/v) of 10 mM sodium phosphate, pH 7.0 , containing 0.1 M NaCl (PBS) with a Polytron homogenizer (Brinkmann Instrument, Westbury, NY). Mucin gel (supernatant) was obtained by centrifugation at 2,000 x g for 10 min at 4 C and redispersed in an equal volume (v/v) of PBS. Homogenization and centrifugation were repeated once. Insoluble mucin was dispersed in 4 volumes (v/v) of 0.1 M sodium phosphate, pH 7.5, containing 0.1 M NaCl and 0.4 M 2-mercaptoethanol and was extracted with stirring under nitrogen for 12 hr at 4 C. Debris was removed by centrifugation at 10,000 x g for 10 min at 4 C and the supernatant was dialyzed (6000 MW cut-off tubing) against several changes of distilled water at 4 C. The final 12 hr of dialysis was against 1 mM sodium phosphate, pH 7.0, containing 0.2 % NaN_3, 10 mM $MgCl_2$ and 5 mM $CaCl_2$. Debris was removed by centrifugation at 10,000 x g for 10 min at 4

C. The supernatant was then digested with DNase (0.025 mg/g wet weight of original mucus) for 6 hr and subsequently treated with trypsin type XI (0.025 mg/g wet weight) at 37 C. After 12 hr, an equivalent amount of trypsin was added and digestion continued for another 12 hr. The solution was dialyzed as above, insoluble debris was removed by centrifugation at 10,000 x g for 10 min and the supenatant was lyophilized.

D. Preparation of K88ab Pili

K88 pili were prepared from E. coli strain with the serotype of O8:K87:K88ab:H19 following the procedure of Stirm et al. (170) with modification. E. coli was cultured overnight in 6 l of Trypticase Soy broth in an incubator (Labline Instruments Inc., Melrose Park, Illinois) at 37 C with shaking at 120 rpm. Cells were harvested by centrifugation at 16,000 x g for 10 min at 4 C and were suspended in 300 ml of 50 mM sodium phosphate buffer, pH 7.3, with 0.15 M NaCl. Cells were homogenized in a Waring blender with a full speed for 1 min. The suspension was centrifuged at 16,000 x g for 15 min at 4 C. The supernatant was collected and stored overnight at 4 C. The precipitate was removed by centrifugation at 16,000 x g for 15 min at 4 C and passed through a membrane filter GA-3 (Gelman Science Inc., Ann Arbor, Michigan) with a pore size of 1.2 μm. K88 pili were precipitated from the supernatant

by adding 1 M acetate to pH 5.3. Pili were collected by centrifugation at 20,000 x g for 15 min at 4 C. The pelleted pili were washed in 50 mM sodium citrate, pH 5.3, and were redissolved in 50 mM sodium phosphate, pH 7.3. The pili solution was filtered (GA-3) and the above procedure was repeated three times. The pili solution was dialyzed and lyophilyzed (170). The pili were resuspended in 0.05 M phosphate, pH 7.3, with 2 M urea and insoluble material was removed by centrifugation at 20,000 x g for 15 min at 4 C. Purified pili were obtained by collecting the excluded volume fraction following gel filtration on Sepharose CL-4B with 0.05 M phosphate, pH 7.3, with 2 M urea. Pili were dialyzed (6000 MW cut-off tubing) against distilled water at 4 C and lyophilized (120).

E. Pili Iodination

The K88 pili were labeled with carrier-free Iodine-125 by a chrolamine-T method (15). An aliquot (0.1 mCi) of $Na^{125}I$ solution was placed into a glass tube (75 mm x 10 mm). The following were added in rapid succesion: A, 10 µl of pili solution (0.5 mg/ml in 0.05 M sodium phosphate, pH 7.5); B, 10 µl of chloramine-T solution (5 mg/ml in 0.25 M sodium phosphate pH 7.5); and C, 100 µl of sodium metabisulphite (4.2 mg/ml in 0.05 M sodium phosphate pH 7.5). After each addition, the solution was mixed thoroughly. The final volume was made to 1.0 ml with KI

solution (2.0 mg/ml in 0.05 M sodium phosphate, pH 7.5 containing 2 % (w/v) bovine serum albumin).

A column of Sepharose CL-4B (12 Cm x 1.6 Cm) was equilibrated with phosphate-serum buffer (0.05 M sodium phosphate, pH 7.5, containing 2 % bovine serum albumin). An aliquot (0.5 ml) of bovine serum albumin solution (0.1 mg/ml in 0.05 M sodium phosphate, pH 7.5) was added to the Sepharose CL-4B column. The entire contents of iodinated pili solution was transferred immediately to the column and eluted with phosphate-serum buffer. Fractions (1 ml) were collected and aliquots (10 µl) were taken for counting in a gamma counter (Gamma 4000 Counting System, Beckman Instrument Inc., Palo Alto, California). ^{125}I-labeled K88 pili were eluted in the void volume fractions, which were collected and stored frozen at - 20 C less than one month prior to use in binding assay. Specific activity of ^{125}I-labeled K88 pili was calculated to be 2.7 µCi/µg.

F. Preparation of Colostrum and Milk Fractions

Colostrum was collected from five sows at the University Swine Center immediately following parturition and milk was collected 15 days later. Milk was also collected from five cows at the University Dairy Center. Skimmilk and cream were prepared by centrifugation (Sorval RC-5B centrifuge) at 12,000 x g for 20 min at room temperature.

Casein was prepared by adjusting the pH of skimmilk to 4.6 with 1 N HCl. Precipitated casein was recovered by centrifugation at 1,000 x g for 10 min at room temperature. Whey (aqueous supernatant) was collected, adjusted to pH 7.3 with 1 N NaOH and saved. Casein was washed twice with distilled water, resuspended in distilled water by adjusting to pH 7.3 with 1 N NaOH, and lyophilized.

Milk fat globule membrane (MFGM) was prepared from cream which had been washed twice with sucrose buffer (SB) solution (0.01 M Tris, pH 7.5, containing 0.28 M sucrose). The washed cream was suspended in SB solution and placed on ice to chill. The cream was homogenized in a Waring blender until visible butter granules appeared and separated. The butter and buttermilk were incubated at 45 C. When butter oil formed at the top, the bottom layer (buttermilk) was removed and centrifuged at 100,000 x g for 60 min at 4 C. Pelleted MFGM was washed once with 50 mM Tris pH 7.5, recovered by centrifugation at 10,000 x g for 15 min at 4 C and stored at -20 C.

G. Preparation of Caseins from Cow Milk

Bovine α_{s1}- and β-caseins were prepared using DEAE-cellulose chromatography (171). Bovine κ-casein was prepared using gel filtration chromatography (181)

H. Binding assay

An aliquot (0.3-0.5 ml) of brush border preparation was suspended in 10 ml of 0.05 M sodium phosphate, pH 7.3, containing 0.1 % bovine serum albumin (PO_4-albumin). The suspension was centrifuged at 16,000 x g for 10 min at 4 C. Pelleted brush border membrane was redispersed in 1 ml of PO_4-albumin. Sufficient lyophilized pili were dispersed in PO_4-albumin to give a final concentration of 0.2 mg protein/ml. Sufficient ^{125}I-pili were included to provide approximately 5 x 10^5 cpm/ml.

The amounts of brush border membrane used in inhibition assay were determined. The amount of brush border membrane added to each assay mixture was varied, while the concentration of pili was held constant at about 7-8 µg/ml. The pili solution was vacuum-filtered through GA-3. Variable amounts (2-130 µl) of brush border suspension were placed in test tubes (45 mm x 10 mm). The volume of the suspension was made up to 140 µl with PO_4-albumin and 10 µl of pili solution was added. The suspension was mixed thoroughly and incubated at room temperature for 1 hr. Aliquots (100 µl) were withdrawn and placed on filter GA-3. Samples were vacuum filtered and washed three times with 1 ml of PO_4-albmin. Membrane filters were placed in tubes and radioactivity determined in a gamma counter. Assay tubes in which PO_4-albumin replaced brush border suspension

served as controls.

I. Inhibition assay

Aliquots of samples (potential inhibitors) were added to binding assay mixtures containing 20 µl of brush border preparations. After PO_4-albmin was added to make a final volume of 140 µl, aliquots (10 µl) of radioactive pili preparations were added. Binding assays were conducted as described above. Controls were conducted in the absence of brush border membrane and/or samples.

J. Statistical Analyses

Quarternary measurements for controls and duplicate measurememts for each level of addition of milk and colostral fractions were made in inhibition assays. A nested analysis of variance and Duncan's multiple range test (166) were used to determine the significance of inhibition of K88ab binding by milk and colostral fractions at each level of addition. Sows and cows were assumed to be random.

K. Electrophoresis

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis described by Laemmli (105) was used. Samples were suspended in 0.008 M Tris, pH 8.9, containing 0.5 % SDS, 0.7 % dithiothreitol, 0.025 mM thymol blue, and 12.5 % glycerol. Samples were heated at 100 C for 5 min and cooled to room temperature prior to electrophoresis at

24 mA (Regulated Power Supply Model 38520, Gelman Instrument Co., Ann Arbor, Michigan) in a model 100 Vertical Gel Electrophoresis Apparatus (Aqueboque Machine and Repair Shop, Aquebogue, New York). Gels were fixed with 12 % trichloroacetic acid for 1 hr, rinsed once with water, and stained in 0.1 % Coomassie blue in 32 % methanol (v/v) and 28 % acetic (v/v) for 1-2 hrs at 60 C. Phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400) were used as low-molecular weight standards. Thyroglobulin (669,000), ferritin (440.000), catalase (232,000), lactate dehydrogenase (140,000), bovine serum albumin (67,000) were used as high molecular weight standards.

Caseins were examined by urea polyacrylamide gel electrophoresis (70). Caseins were suspended in a solution containing 0.03 % Tris, 0.144 % glycine, 5 % (v/v) 2-mercaptoethanol, and 0.002 % bromophenol blue. After addition of sucrose, samples were subjected to electrophoresis at 250 volts. Gels were stained with 1 % amido black in 7 % acetic acid (v/v) and destained with 7 % acetic acid as described above.

L. Western blot

Samples were first subjected to electrophoresis and were then electroblotted onto nitrocellulose papers

in 25 mM Tris-HCl - 192 mM glycine, pH 8.3, containing 20 % methanol at 60 V for 3 hrs (Trans-blot cell, Bio-Rad, Richmond, California). Nitrocellulose papers were immersed in 3 % bovine serum albumin in PBS (20 mM sodium phosphate, pH 7.5, containing 140 mM NaCl) and incubated for 1 hr. Subsequently, nitrocellulose papers were incubated in K88 pili (0.5 μg/ml) or concanavalin A (1.0 μg/ml), anti-K88 serum (1:2,000) or anti-concanavalin serum (1:2000), and anti-rabbit IgG-horseradish peroxidase conjugate (1:2,500) in PBS containing 1 % bovine serum albumin and 0.05 % Tween-20 for 1 hr. Each step was followed by washing twice in PBS containing 0.05 % Tween-20 once for 5 min. After final step filters were further washed in PBS for 5 min once and placed in 83 μg/ml o-dianisidine in 0.2 M Tris, pH 7.5, containing 0.01 % hydrogen peroxide for 10 to 20 min. Reaction was stopped by washing with water. In control assays, PBS containing 1 % bovine serum albumin and 0.05 % Tween-20 was used.

M. Dot Blot Assay

Proteins or brush border membrane (0.5 μg protein) in 20 mM phosphate, pH 7.3, containing 140 mM NaCl were placed on nitrocellulose paper. The paper was thoroughly dried and subjected to the procedure described above for Western blot assay.

N. Periodate Treatment

Brush border membrane and mucus were incubated in 1 ml of 0.2 M sodium acetate buffer, pH 4.5, containing 0.01 M sodium metaperiodate at 37 C for 1 hr (28)). Treated brush border membrane was centrifuged at 10,000 x g for 15 min and washed twice with 0.1 M PO_4 buffer, pH 7.5. Treated mucus was added to 5 ml of 0.1 M PO_4 buffer, pH 7.5, dialyzed in distilled water and lyophilized. Controls were incubated in 0.2 M sodium acetate, pH 4.5, containing 0.01 M sodium iodate at 37 C for 1 hr.

O. Carbamylation

Brush border membrane (5 mg protein/ml) was dispersed in 0.5 M sodium borate buffer, pH 8.0, containing 0.03 mM PMSF (phenylmethylsulfonyl fluoride) and 0.01 % EDTA. Potassium cyanate (20 mg/mg protein) was added to the suspension, which was incubated at 37 C for 4 hrs and dialyzed against 10 mM sodium borate buffer, pH 8.0, containing 0.15 M NaCl for 24 hr and against distilled water overnight at 4 C. A control was treated in the same manner but without adding potassium cyanate. The procedure was based on the methods reported by Weisgraber (177).

P. Pronase digestion

Pronase digestion of brush border membrane was performed by the method described by Cummings and Kornfeld (32) with modification. Brush border membrane was

suspended in 0.1 M Tris-HCl. pH 8.0, containing 1 mM $CaCl_2$, homogenized with a few strokes in a Potter-Elvehjem tissue grinder, and was digested with 2 % (w/w) pronase at 50 C after addition of 1 % toluene. Pronase was predigested at 50 C for 1 hr to inactivate possible exoglycosidase. After 24 hrs additional pronase (2 %) was added and digestion allowed to continue for another 24 hrs. Digest was boiled for 5 min, centrifuged at 10,000 x g for 30 min, desalted on a column of Sephadex G-15 (1.5 Cm x 50 Cm) in 10 mM pyridine-acetate buffer (pH 5.0), and lyophilized.

Q. Biochemical and Immunological Analysis

Protein concentration was determined by colorimetric method of Lowry et al. (112) using serum albumin (5 mg/ml) as standard. Alkaline phosphatase activity associated with intestinal brush border membrane preparation and homogenates was determined by the method of Murer et al. (126). Activity was determined with 5.5 mM p-nitrophenol phosphate as substrate in glycine buffer (0.5 M glycine, pH 10.5, 20 mM $MgSO_4$, and 2.5 mM $ZnSO_4$). Neutral sugar was determined by phenol-sulfuric acid method (41) with mannose (20 µg/ml) as standard. Double immunodiffusion plate was performed by following the procedure described by Campbell (21).

CHAPTER FOUR

RESULTS

A. CHARACTERIZATION OF RECEPTORS FOR K88AB PILI IN PIG SMALL INTESTINAL BRUSH BORDER MEMBRANE AND MUCIN

1. Preparation of K88ab Pili and Anti-K88ab Serum

K88ab pili were isolated from a strain of Escherichia coli with the serotype of O8:K87,K88ab:H19. The K88ab pili preparation appeared highly purified in SDS-polyacrylamide gel electrophoresis (Fig. 1.1). The molecular weight of K88ab subunit was 25,600, which is similar to reported molecular weight values (23,500-26,000) based on SDS gel electrophoresis (118) but slightly lower than the calculated molecular weight (27,540) (97). K88ab pili were dissociated into subunits in the presence of 6 M guanidium hydrochloride which were eluted in the included volume on gel filtration chromatography with Sepharose CL-6B (results not shown). The anti-K88ab serum was prepared from a rabbit which was immunized with the isolated K88ab pili. The isolated K88ab and K88ac pili reacted with anti-K88ab and anti-K88ac serum in double immunodiffusion assay (Fig. 1.2). Formation of spurs indicated that partial identity exists between K88ab and K88ac pili (Fig. 1.2).

2. Inhibition Assay

The amount of K88ab pili bound by brush border membrane was determined by subtracting the amount retained

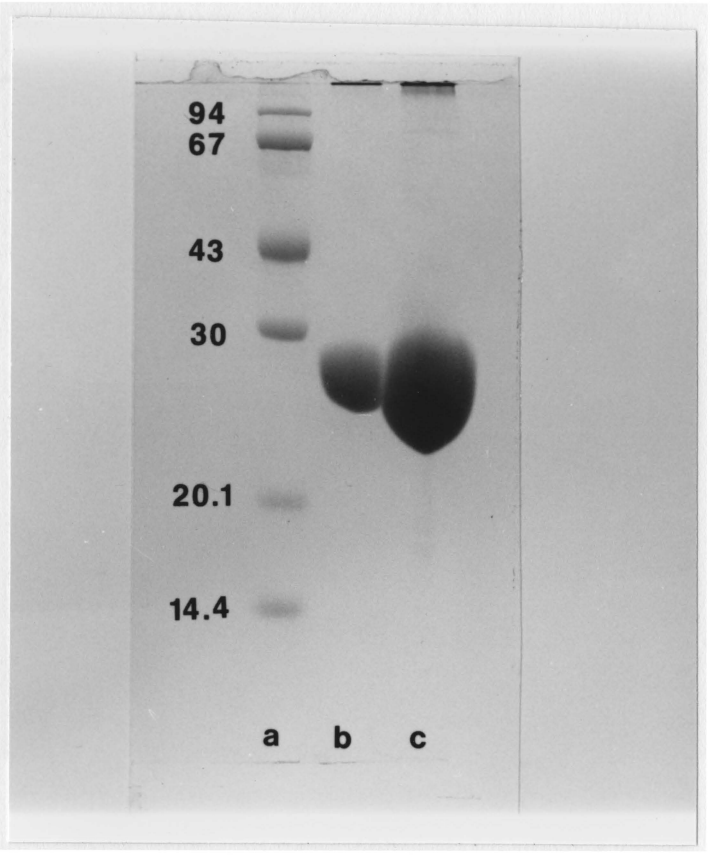

Figure 1.1. The isolated K88ab pili in SDS-polyacrylamide gel electrophoresis. (a) molecular weight markers (top to bottom) phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400). The numbers at the left side of the figure are molecular weights x 10^{-3}. (b) K88ab pili (50 μg), (c) K88ab pili (250 μg).

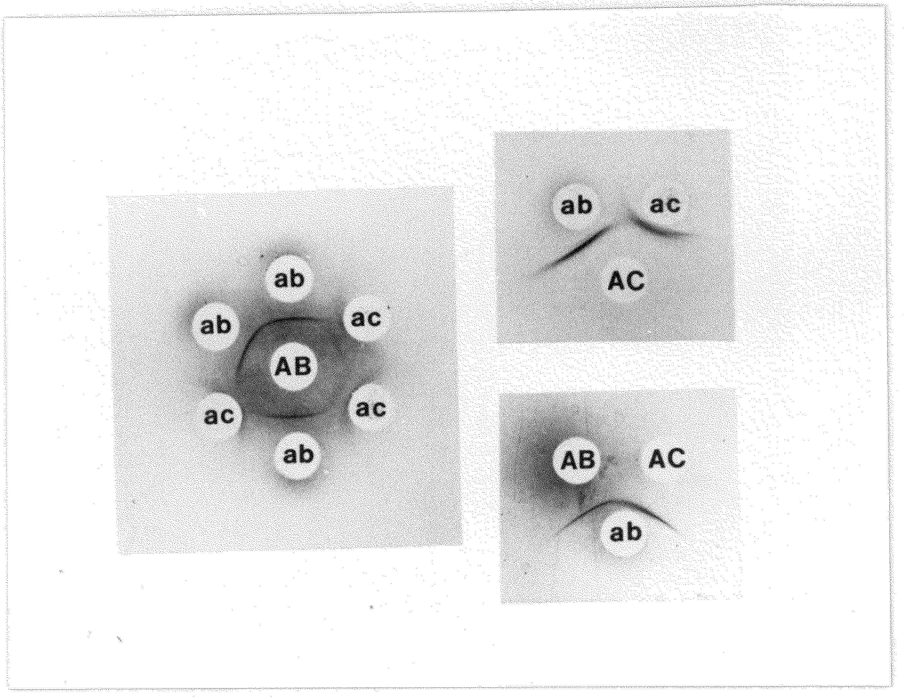

Figure 1.2. Double immunodiffusion assay. K88ab pili (ab); K88ac pili (ac); anti-K88ab serum (AB); anti-K88ac serum (AC).

by filter from the total amount bound. The amounts of pili retained by the filter in inhibition assay were less than 15 %. K88ab pili bound by brush border membrane increased linearly, as the concentration of brush border membrane in the assay mixture increased, up to 0.5 mg protein/ml. When brush border membrane concentration was more than 0.5 mg/ml, the amount of K88ab pili bound leveled off (Fig. 1.3). Brush border membrane concentration used in the inhibition assay was 0.3 mg/ml, which was in the range of linear increase.

3. Inhibition of K88ab Binding by Carbohydrates

The effect of various monosaccharides and methyl-derivatives of monosaccharides on binding of K88ab pili to brush border membrane was examined in the inhibition assay (Table 1.1). Addition (100 mM) of galactosamine, glucosamine, and mannosamine resulted in strong inhibition of K88ab binding, while addition of similar levels (100 mM) of galactose, mannose, glucose, fucose, N-acetylgalactosamine, and N-acetylglucosamine had little effect. Low levels (10 mM) of galactosamine and glucosamine also inhibited binding (Table 1.1). In addition, low levels (10 mM) of α - and β-methylglucose, α - and β-methylgalactose, α -methylmannose, α-methyl-N-acetylgalactosamine, and β-methyl-N-acetyl-glucosamine had no effect on binding of K88ab pili (results not shown).

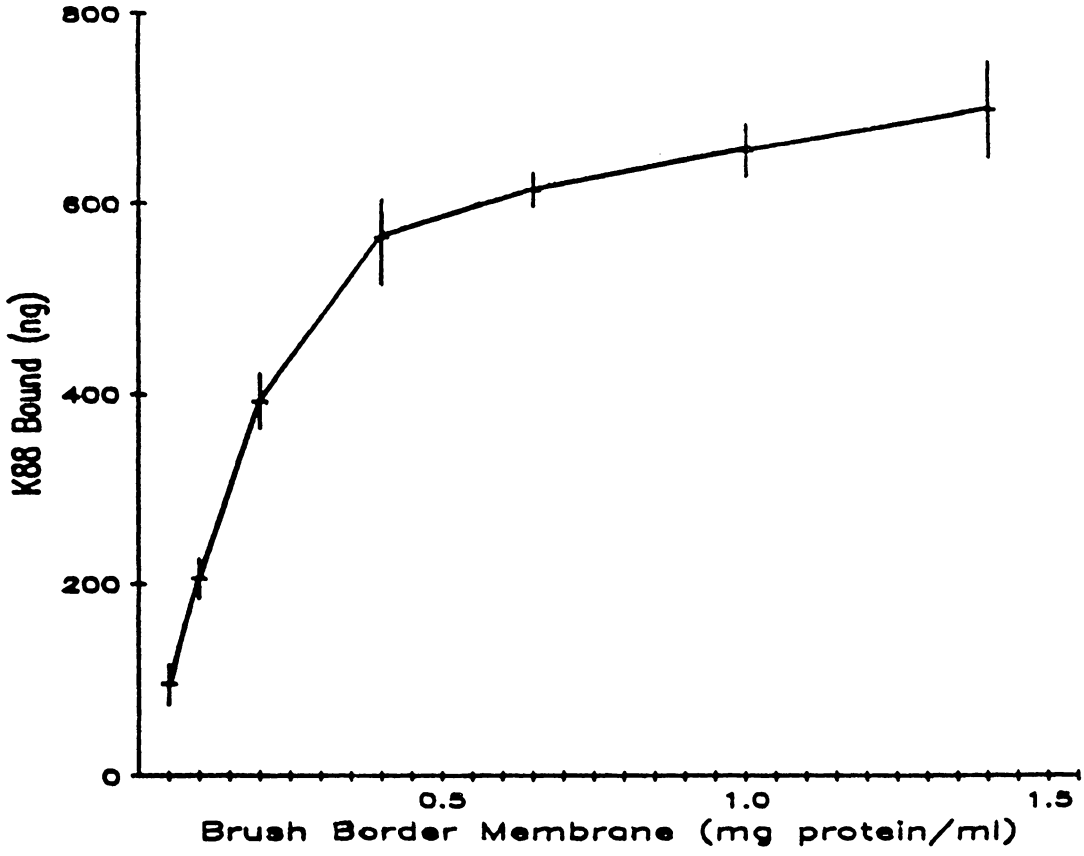

Figure 1.3 Binding of K88ab pili by various concentrations of brush border membrane.

Table 1.1. Effect of carbohydrates on binding of K88ab pili by brush border membrane prepared from a piglet[a]

Assay mixture		K88ab bound to brush border membrane
Added Component	Amount	(%)
No addition		100.0 ± 13.6
Mannose	100 mM	82.1 ± 7.0
Glucose	100 mM	89.0 ± 18.9
Galactose	100 mM	80.5 ± 12.8
Fucose	100 mM	95.9 ± 6.7
Mannosamine	100 mM	18.5 ± 4.3
Glucosamine	10 mM	46.7 ± 13.7
	100 mM	2.9 ± 10.5
Galactosamine	10 mM	24.5 ± 5.0
	100 mM	0.1 ± 2.1
N-Acetylglucosamine	100 mM	85.7 ± 10.3
N-Acetylgalactosmine	100 mM	74.8 ± 7.3

a Two assays were performed and duplicate measurement were made for each assay.

4.K88ab Receptor in Brush Border Membrane

Western blot assay was used to identify and characterize polypeptides in brush border membrane which bind K88ab pili. Brush border membrane from a 21-day-old piglet was subjected to SDS-polyacrylamide gel electrophoresis. The major polypeptides had molecular weights of 94,000, 61,500, 50,000, and 13,500 (Fig. 1.4 (a)). Immediately after electrophoresis, polyacryamide gel were electrophoretically blotted in order to transfer and immobilize polypeptides to nitrocellulose paper. After saturation with bovine serum albumin, binding of K88ab pili by receptor polypeptides was detected by incubating nitrocellulose paper sequentially with K88ab pili, anti-K88ab rabbit serum, anti-rabbit IgG-peroxidase conjugate, and chromogenic substrate, o-dianisidine. The major receptor polypeptides for K88ab pili had molecular weights of 61,500 and 57,000 (Fig. 1.4 (b)). Additionally, numerous minor receptor polypeptides across a wide range of molecular weights could be detected. In control assays, incubation with K88ab pili was omitted to determine whether anti-K88ab rabbit serum or anti-rabbit IgG-peroxidase conjugate interact with polypeptides on the nitrocellulose paper. Although no interaction was detected, some Western blot assays (Fig. 1.5, 1.6, and 1.7) contained several dotted bands with a molecular weight of approximately

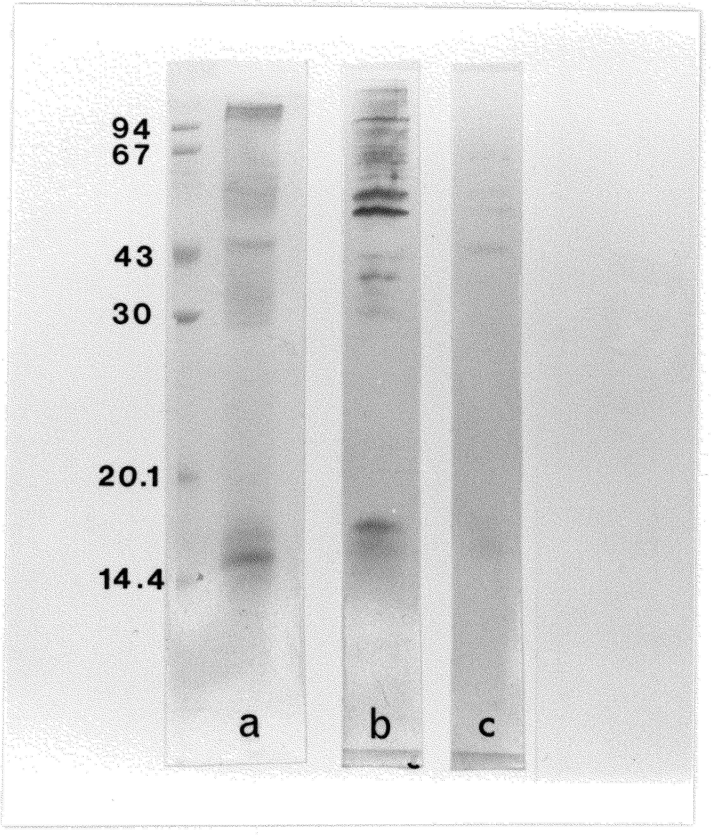

Figure 1.4. Binding of K88ab pili by polypeptides of brush border membrane from a 21-day-old pig as detected by Western blot. (a) SDS-polyacrylamide gel electrophoresis of brush border membrane; (b) Western blot showing binding of K88ab pili; (c) Western blot showing binding of K88ab pili in the presence of D-galactosamine (20 mM). The numbers at the left side of the figure are molecular weight x 10^{-3} of molecular weight markers described in Fig. 1.1.

63,000. Apparently, contaminants in the dispersing buffer used in SDS-gel electrophoresis were responsible for binding of antibodies. Additionally, some brush border membrane preparations contained major receptors with molecular weights of 15,500 and 13,500 (Fig. 1.6). D-galactosamine was added to K88ab solution to determine whether binding by receptor polypeptides in Western blot was the same reaction as monitored in inhibition assay. Addition of D-galactosamine resulted in almost complete inhibition of K88ab binding by receptor polypeptides in brush border membrane (Fig. 1.4 (c)).

5. K88ab Receptor in Mucin

Western blot was also used to identify receptors for K88ab pili in mucin isolated from a 2-day-old piglet. Major polypeptides present in isolated mucin had molecular weights of 26,500, 24,000 and 15,500 (Fig. 1.5 (a)). The major receptor polypeptides detected had a molecular weight of 26,500 (Fig. 1.5 (b)), while several minor receptor polypeptides (M.W. 35,000 and 15,500) were also present. Preparations of crude mucus also contained a major receptor polypeptide with a molecular weight of 27,500 and numerous minor high-molecular-weight receptor polypeptides. These minor polypeptide receptors in crude mucus had patterns similar with those detected in brush border membrane (results not shown). Addition of D-galactosamine to K88ab

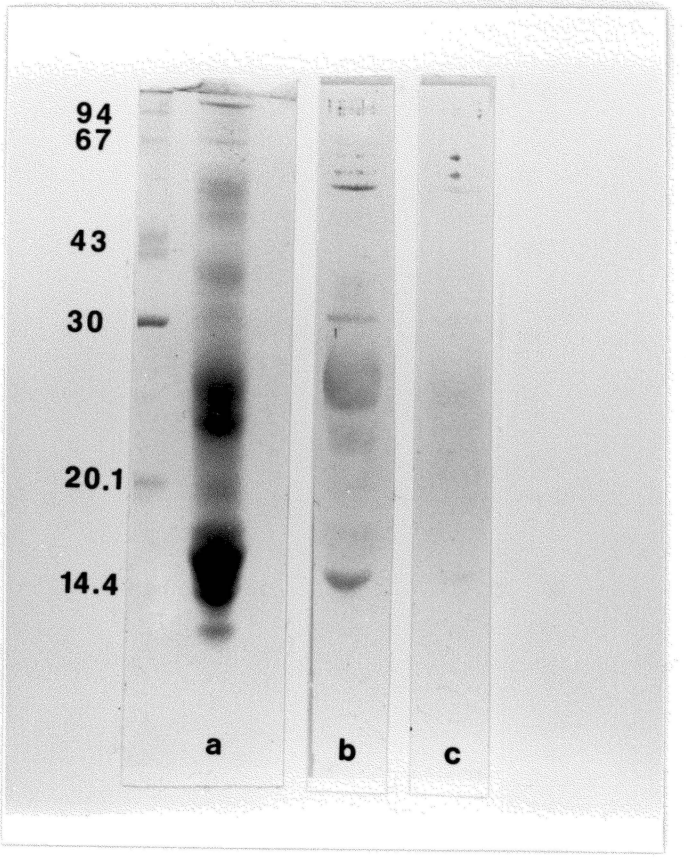

Figure 1.5. Binding of K88ab pili by polypeptides of mucin from a 2-day-old pig as detected by Western blot. (a) SDS-polyacrylamide gel electrophoresis of mucin; (b) Western blot showing binding of K88ab pili; (c) Western blot showing binding of K88ab pili in the presence of D-galactosamine (20 mM). The numbers at the left side of the figure are molecular weight x 10^{-3} of molecular weight markers described in Fig. 1.1.

pili solution also inhibited binding of K88ab by receptor polypeptides in mucin (Fig. 1.5 (c)).

6. Effect of Periodate Treatment on Receptor Polypeptides

Treatment of brush border membrane and mucin with sodium metaperiodate was used to determine whether carbohydrates were involved in their ability to bind K88ab. Treatment with sodium iodate served as control. Binding of concanavalin A by receptor polypeptides in treated mucin was also used as a control to determine whether the periodate treatment was sufficiently strong to complete oxidation of carbohydrates. Binding of concanavalin A was detected by substituting concanavalin A and anti-con A serum for K88ab pili and anti-K88ab serum in the Western blot.

After periodate treatment, polyacrylamide gel patterns obtained for brush border membrane and mucin contained fewer polypeptides than did controls (Fig. 1.6. A(b and g)). A portion of both brush border membrane and mucin polypeptides did not even penetrate into the separating gel (indicated by * in Fig. 1.6). Periodate treament decreased, but did not completely destroy, binding of K88ab by receptor polypeptides in the brush border membrane and mucin, (Fig. 1.6 B). However, polypeptides in the periodate-treated brush border membrane with molecular weights of 15,500 and 13,500 still strongly bound K88ab

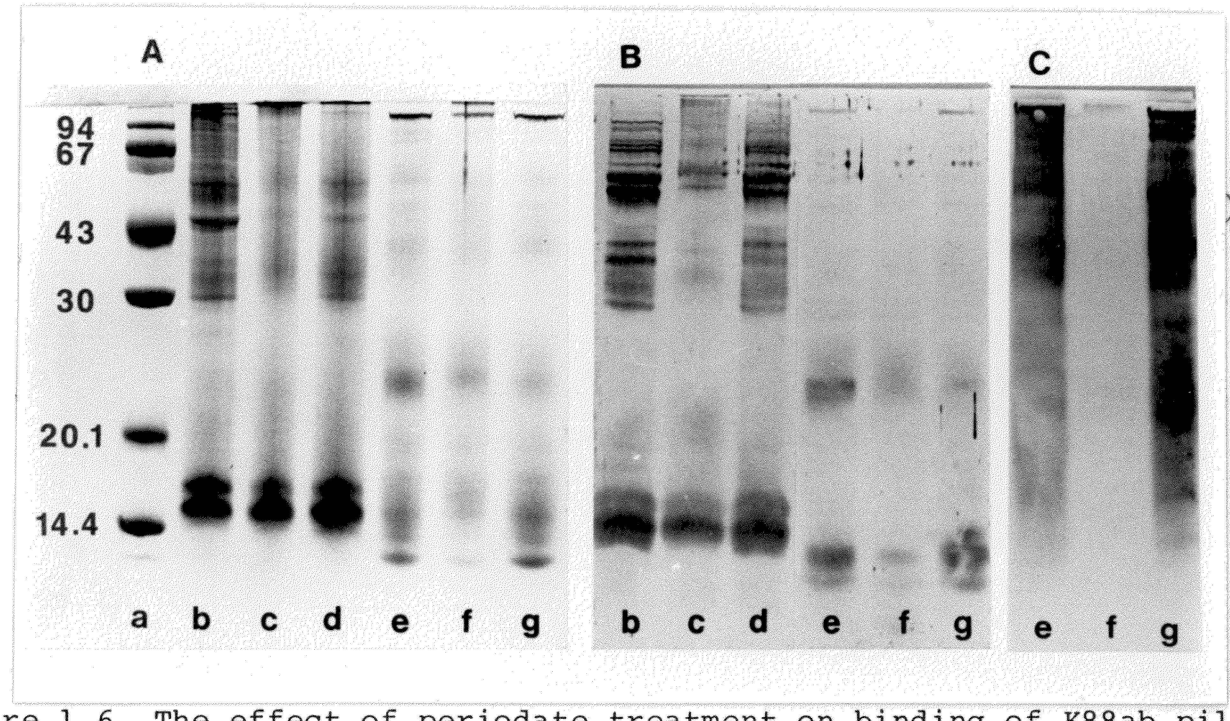

Figure 1.6. The effect of periodate treatment on binding of K88ab pili as detected by Western blot. (A) SDS-polyacrylamide gel electrophoresis; (B) Western blot showing binding of K88ab; (C) Western blot showing binding of concanavalin A. (a) molecular weight markers; (b) brush border membrane without treatment; (c) brush border membrane treated with periodate; (d) brush border membrane treated with iodate; (e) mucin without treatment; (f) mucin treated with periodate; (g) mucin treated with iodate. The numbers at the left side of the figure correspond to molecular weight x 10^{-3} of molecular weight markers described in Fig. 1.1.

pili. The residual binding intensity by other receptor polypeptides in the periodate-treated brush border membrane and mucin was similar to the staining intensity of the corresponding polypeptides. However periodate treatment completely destroyed receptors for concanavalin A in mucin (Fig. 1.6 C).

7. Inhibition of K88ab Binding by Brush Border Membrane Glycopeptide

In order to isolate and identify glycopeptides containing receptors for K88ab pili, brush border membranes prepared from two different pigs were extensively digested with pronase. The pronase solutions were pre-digested before addition to the brush border membrane suspensions to destroy exoglycosidase activity which may cleave nonreducing terminal residues from oligosacharides. The glycopeptide preparations were used in inhibition assay to determine their effect on binding of K88ab pili by brush border membrane. Addition of the two glycopeptide preparations had no effect on binding of K88ab (Table 1.2). The dry solid of the glycopeptide preparation added to the assay mixtures was approximately the same amount as brush border membrane protein (80 μg/150 μl).

8. Effects of Carbamylation on K88ab Receptor

Carbamylation of brush border membrane with cyanate was used to determine if amino groups were involved in

Table 1.2. Effect of brush border membrane glycopeptide preparations from two different piglets on binding of K88ab pili by brush border membrane prepared from a piglet[a]

Assay mixture		
Component membrane added	Amount	K88ab pili bound to brush border
	µg neutral sugar 150 µl	µg solid 150 µl	(%)
No addition			100.0 ± 17.9
Preparation 1	15	-	96.4 ± 7.8
Preparation 2	15	78	110.7 ± 10.7

a Duplicates for no addition and preparations were determined.

binding of K88ab pili. Incubation of brush border membrane in borate buffer but without cyanate was used as control. Additionally, binding of concanavalin A in Western blot was used to determine whether carbamylation modified the carbohydrate structure. PMSF and EDTA were included to prevent proteolysis of brush border membrane during cyanate treatment.

Carbamylation of brush border membrane produced a decline in intensity of polypeptides (Fig. 1.7 A(c)) detected by SDS-gel electrophoresis compared to controls (Fig 1.7 A(b and d)), although identical amounts of protein was loaded into each lane. Carbamylation was effective in completely destroying the ability of receptor polypeptides in brush border membrane to bind K88ab pili. The dotted bands which migrate at molecular weight 63,000 (Fig 1.7 B(c)) were due to contaminants in dispersing buffer, as mentioned above. However, similar treatment produced only slight alterations in the binding patterns obtained for concanavalin A (Fig 1.7 C). No changes could be detected in binding patterns of K88ab pili (Fig 1.7 B(d)) and concanavalin A (Fig 1.7 C(d)) by control brush border membrane treated in the absence of cyanate.

9. K88ab Receptor Patterns of Brush Border Membrane and Mucin from Pigs of Various Ages.

The polypeptide and receptor patterns for brush border

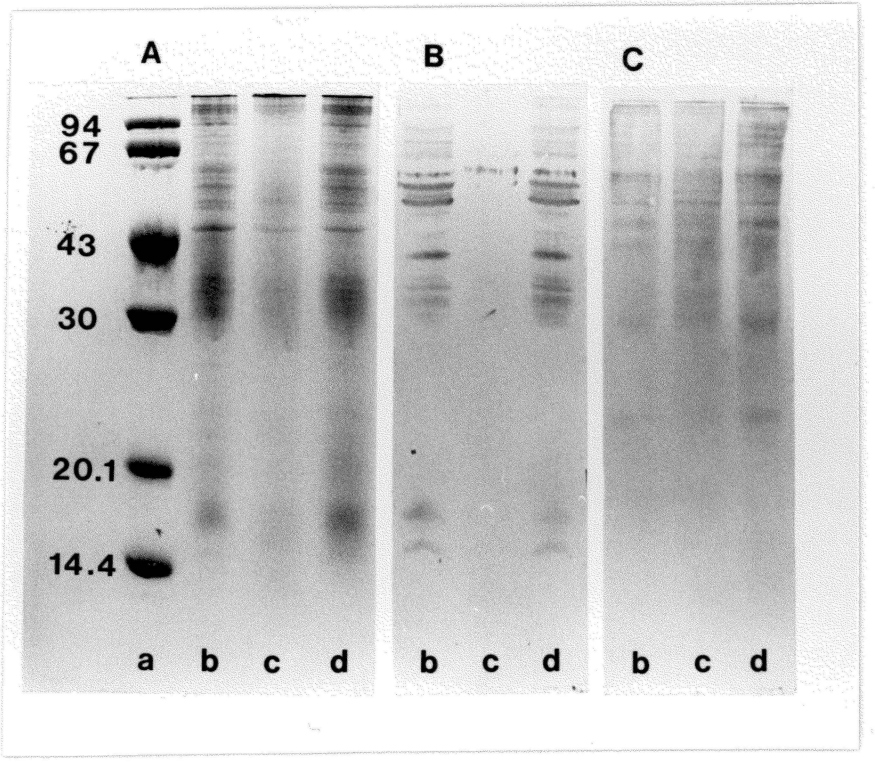

Figure 1.7. The effect of carbamylation of brush border membrane with cyanate on binding of K88ab pili. (A) SDS-polyacrylamide gel electrophoretic pattern; (B) binding of K88ab pili; (C) binding of concanavalin A. (a) molecular weight markers; (b) Brush border membrane without treatment; (c) brush border membrane treated with cyanate; (d) brush border membrane treated without cynate. The numbers at the left side of the figure correspond to molecular weight x 10^{-3} of molecular weight markers described in Fig. 1.1.

membranes prepared from 2-, 21-, and 42-day-old piglets and adult hogs were compared (Fig. 1.8). Alhough the polypeptide patterns obtained from brush border membrane from 2-day-old piglets were somewhat different from the others (Fig. 1.8 A), no major consistent differences could be detected. The major receptor polypeptides (M.W. 61,500 and 57,000) mentioned previously (Fig. 1.4 b) were also present in all preparations of brush border membranes from 2-, 21-, and 42-day-old piglets. Among brush border membrane preparations obtained from four hogs, two possessed both major receptor polypeptides (Fig 1.8 B (e)). Preparations of brush border membranes from the other two hogs contained either one (M.W. 57,000) of two major receptor polypeptides or no major receptor polypeptides (results not shown). Various differences could be detected in patterns of minor receptors among brush border membranes prepared from pigs of different ages.

Receptor patterns for mucin prepared from 2-, 21-, and 42-day-old piglets and hogs were also compared (Fig. 1.9). Mucin from 2-day-old piglets contained a major receptor polypeptide (M.W. 26,500) as well as minor polypeptides (Fig. 1.9 B (b)). However, the major receptor polypeptide appeared to be one of the minor receptor polypeptides in mucin obtained from 21- and 42-day-old piglets and hogs. Additionally, some minor receptor polypeptides detected in

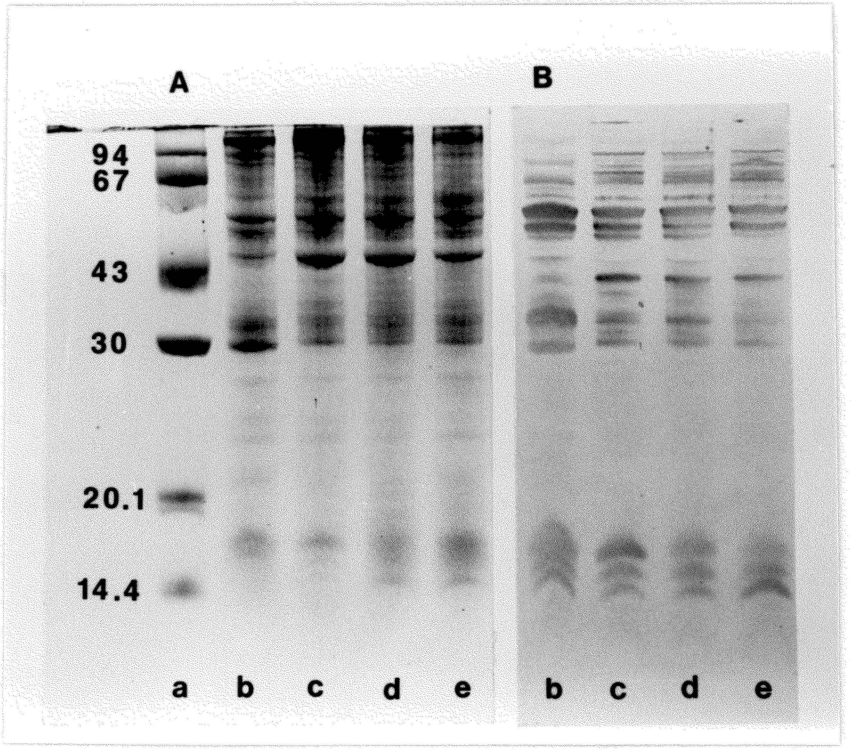

Figure 1.8. SDS-polyacrylamide gel electrophoretic patterns (A) and binding of K88ab (Western blot) (B) by brush border membrane polypeptides prepared from pigs of various ages. (a) molecular weight markers. Brush border membrane preparations b, c, d, and e, were from 2-, 21-, and 42-day-old piglets, and a hog, respectively. The numbers at the left side of the figure correspond to molecular weight x 10^{-3} of molecular weight markers described in Fig. 1.1.

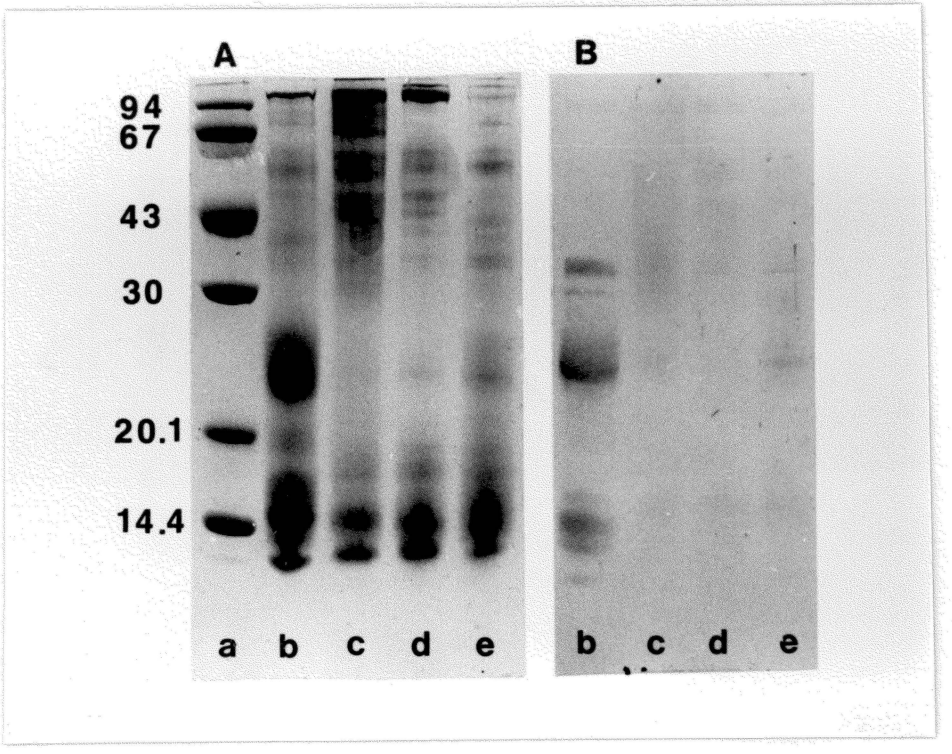

Figure 1.9. SDS-polyacrylamide gel electrophoretic patterns (A) and binding of K88ab (Western blot) (B) by mucin polypeptides prepared from pigs of various ages. (a) molecular weight marker. Mucin preparations b, c, d, and e, were from 2-, 21-, and 42-day-old piglets, and a hog, respectively. The numbers at the left side of the figure correspond to molecular weight x 10^{-3} of molecular weight markers described in Fig. 1.1.

mucin from the 2-day-old piglet also were not present in mucin prepared from older pigs. Two other sets of mucin preparations from pigs of various ages showed similar patterns, while in a fourth, set receptor polypeptides decreased gradually as pigs grew older.

B. IDENTIFICATION OF PROTEINS IN MILK AND COLOSTRUM WHICH BIND K88AB PILI

1. Inhibition of K88ab Binding

Most components prepared from sow colostrum and milk had significant inhibitory effects on binding of K88ab pili by brush border membrane (Table 2.1). Whole colostrum and milk were more inhibitory than skim colostrum and milk, but the differences were not significant. Milk fat globule membrane (MFGM) not only competed with brush border membrane, but K88ab pili bound by MFGM were also retained by filters.

Double immunodiffusion plate was used to determine the presence of of IgG, IgA, and IgM in MFGM, casein, and whey from both sow milk and colostrum as well as to detect anti-K88ab antibody (results not shown). Whey prepared from colostrum and milk contained all three Igs. MFGM prepared from colostrum contained IgG and IgM, while IgG and IgA could be detected in casein from colostrum. Immunoglobulins could not be detected in either MFGM or casein prepared from milk. No anti-K88ab antibody could be

Table 2.1. Effect of sow colostrum and milk fractions on binding of K88ab by pig brush border membrane.

Component added	Amount µg protein 150 µl	Binding (%)
No addition		100.0
Colostrum		
Whole colostrum	200	46.6**
	600	18.4**
Skim colostrum	200	52.6**
	600	25.7*
MFGM	20	62.4**
	60	21.4*
Casein	100	61.0**
	300	41.7
Whey	100	77.8**
	300	44.7
Milk		
Whole milk	200	50.0**
	600	36.2**
Skim milk	200	55.3*
	600	58.4**
MFGM	20	38.5**
	60	13.6**
Casein	100	18.2**
	300	43.7*
Whey	100	68.4**
	300	53.9

* Difference from no addition is significant ($P<.05$).
** Difference from no addition is significant ($P<.01$).

detected in any preparation of colostrum from the 5 different sows used in this study.

Skim milk and casein prepared from cow's milk had the ability to inhibit binding of K88ab to brush border membrane (Table 2.2). Although whole milk was somewhat inhibitory, differences were not significant at the 0.05 level. Binding of K88ab by MFGM prepared from cow's milk was one fourth of the level bound by MFGM from sow milk (data not shown).

Whey from sow colostrum is abundant in immunoglobulins. In order to determine the role of immunoblobulins in inhibition of K88ab binding, whey from sow colostrum was further fractionated into five peaks by gel filtration on Sephadex G-200 (Fig. 2.1). In inhibition assay (Table. 2.3), fraction A had the greatest ability to inhibit binding of K88ab by brush border membrane. Fractions B, E, and F were only marginally effective. Only fraction A contained all three immunoglobulins. Fractions B and C contained IgG.

2. Identification of K88ab-Binding Protein in Milk and Colostrum

MFGM prepared from sow milk and colostrum contained two major polypeptides (155,000, and 68,000) which had ability to bind K88ab pili (Fig. 2.2). Minor K88ab-binding polypeptides present had molecular weights of 58,000 and

Table 2.2. Effect of cow milk fractions on binding of K88ab by pig brush border membrane.

Component added	Amount µg protein 150 µl	Binding (%)
No addition		100.0
Milk		
Whole milk	200	62.5
	600	61.0**
Skim milk	200	16.0**
	600	29.5
MFGM	20	88.9
	60	70.1**
Casein	100	39.2**
	300	41.5
Whey	100	86.7
	300	71.2

** Difference from no addition is significant ($P<.01$).

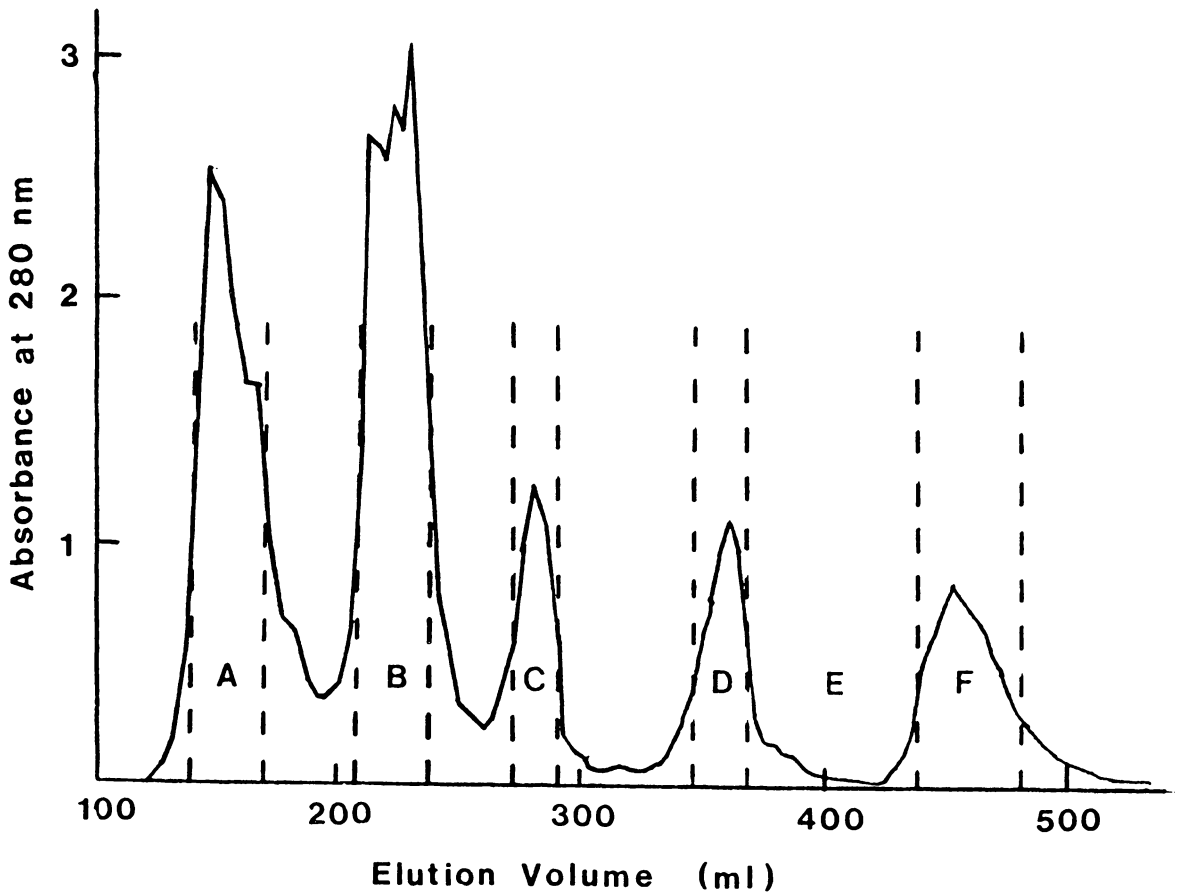

Figure 2.1. Gel filtration pattern obtained for colostral whey on Sephadex G-200 (2.6 by 80 Cm) after elution with 0.05 M ammonium acetate.

Table 2.3. Effect of sow colostral whey fractions on binding of K88ab by pig brush border membrane.

Component added	Amount μg protein 150 μl	Binding (%)
No addition		100.0 ± 10.1
A	100	47.7 ± 8.7
	300	38.0 ± 14.9
B	100	77.2 ± 5.8
	300	33.0 ± 8.2
C	100	108.6 ± 6.7
	300	81.8 ± 4.5
D	100	107.0 ± 5.7
	300	90.4 ± 5.3
E	100	93.9 ± 8.6
	300	49.9 ± 0.7
F	100	84.3 ± 3.8
	300	45.3 ± 3.9

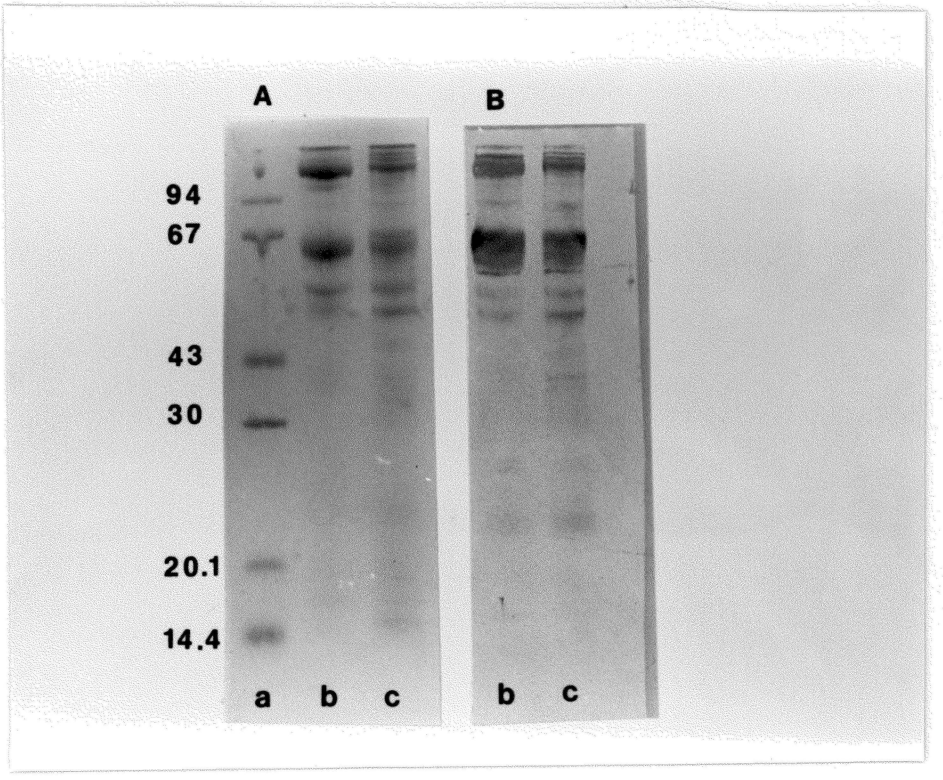

Figure 2.2. SDS polyacrylamide gel electrophoresis (A) and Western blot (B) of MFGM. (a) molecular weight markers, (b) sow colostrum, (c) milk. The numbers at the left side of the figure correspond to molecular weight x 10^{-3} of molecular weight markers described in Fig. 1.1.

53,000. In MFGM prepared from another sow, one minor polypeptide (M.W. 58,000) appeared as an additional major K88ab-binding polypeptide. Most major polypeptides present in MFGM had a tendency to bind K88ab pili. Caseins from sow and cow milk were examined for their ability to bind K88ab pili by Western blot after urea-gel electrophoresis (Fig. 2.3). Sow casein contained only a single polypeptide which could bind K88ab. Major caseins present in cow milk had strong binding activity.

Fractions obtained following gel filtration (Fig. 2.1) of sow colostral whey were subjected to SDS-electophoresis in the presence of dithiothreitol and Western blot (Fig. 2.4). The major polypeptides present in colostral whey had molecular weights of 69,000, 57,000, 26,000 (doublet), and 19,000. Fractions A and B contained two K88ab-binding polypeptides (M.W. 57,000 and 26,000), while fraction C contained one (M.W. 71,000). One polypeptide with less ability to bind K88ab pili had a molecular weight of 69,000. These same fractions from colostral whey were also examined in the absence of dithiothreitol (Fig. 2.5). The major polypeptides present in colostral whey had molecular weight of 170,000, and 67.000. One major polypeptide (M.W. 170,000) in fractions A and B had the ability to bind K88ab pili. Some minor K88ab-binding polypeptides could be detected just below the major polypeptide band. No

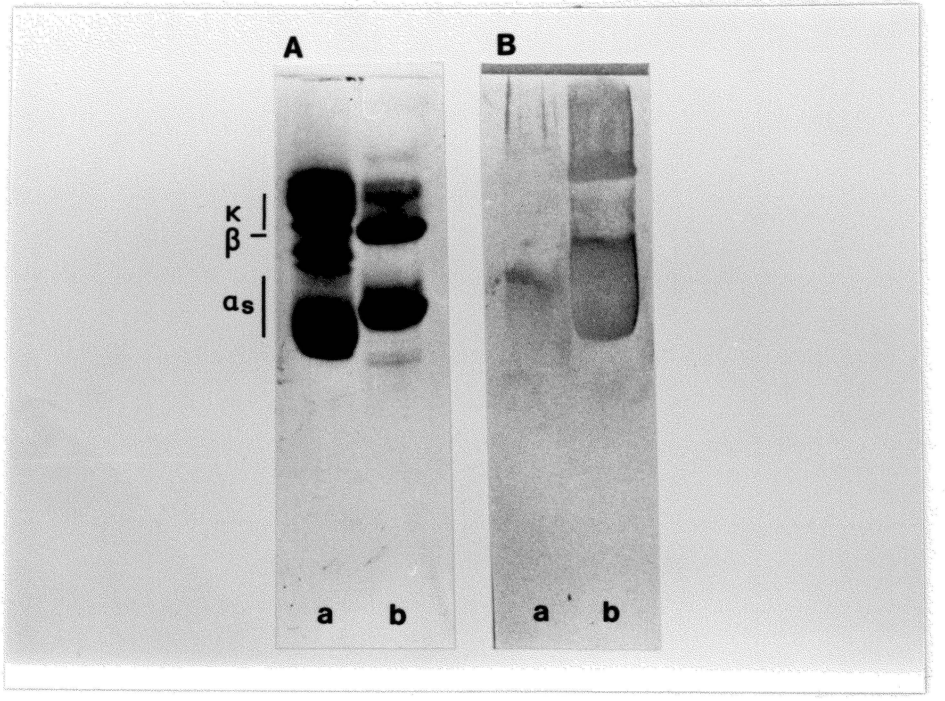

Figure 2.3. Urea gel electrophoresis (A) and Western blot (B) of sow casein (a) and cow casein (b). The Greek letters at the left side indicate the locations of α_{s1}-, β-, and κ-casein from cow milk based on the review by Swaisgood (170).

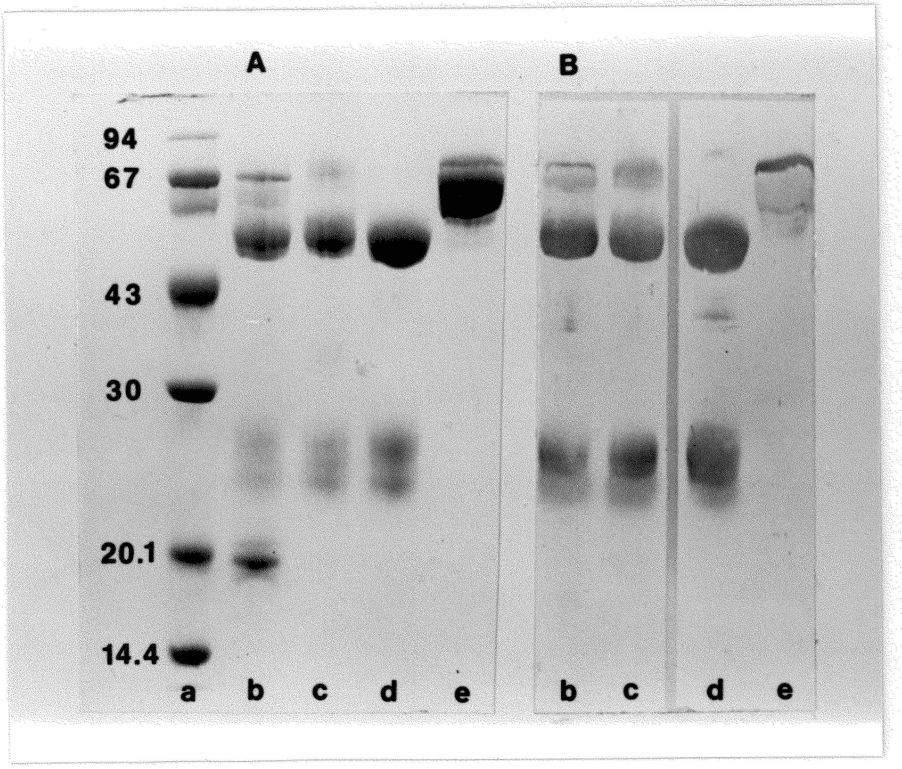

Figure 2.4. SDS polyacrylamide gel electrophoresis (A) and Western blot (B) of whey fractions from gel fitration of sow colostral whey (Fig. 2.1). The samples were reduced with dithiothreitol. (a) molecular weight markers (b) colostral whey, (c) fraction A, (d) fraction B, (e) fraction C. The numbers at the left side of the figure correspond to molecular weight x 10^{-3} of molecular weight markers described in Fig. 1.1.

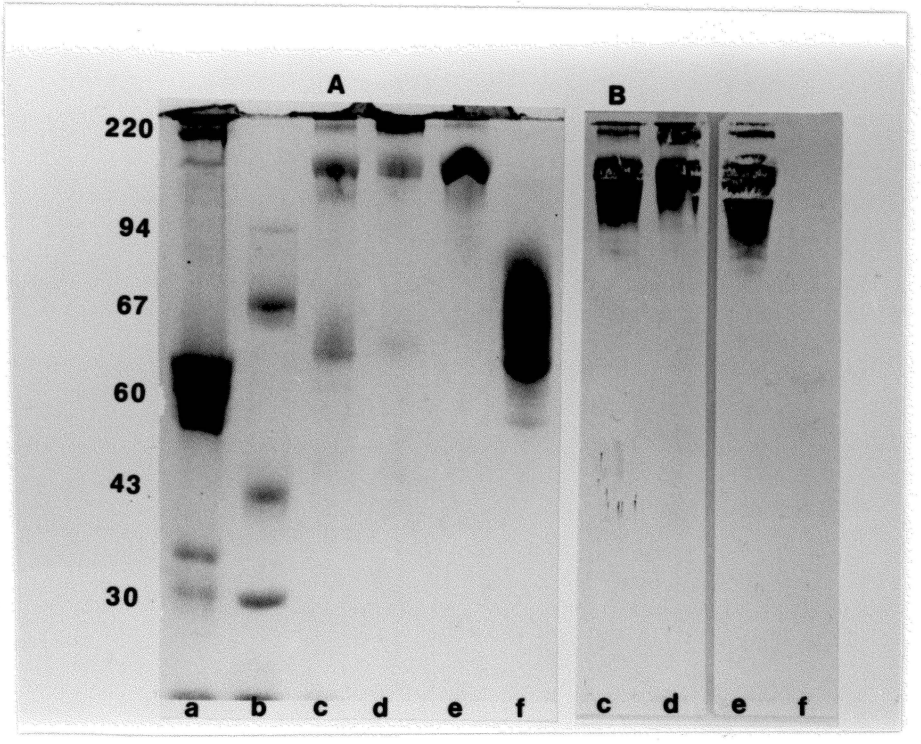

Figure 2.5. SDS polyacrylamide gel electrophoresis (A) and Western blot (B) of whey fractions from gel fitration of sow colostral whey (Fig. 1). The samples were not reduced with dithiothreitol. (a and b) molecular weight markers (top to bottom) ferritin subunit (220,000), phosphorylase b (94,000), bovine serum albumin (67,000), catalase subunit (60,000), ovalbumin (43,000), carbonic anhydrase (30,000), The numbers are at the left side of the figure correspond to molecular weight x 10^{-3} of moleuclar weight marker proteins. (c) colostral whey, (d) fraction A, (e) fraction B, (e) fraction C.

polypeptides in fraction C appeared to bind K88ab pili.

Few polypeptides in whey and milk fat globule membrane from cow milk showed significant binding of K88ab in Western blot (results not shown). Caseins which co-migrated with α_{s1}-casein had the ability to strongly bind K88ab (Fig. 2.6 (a and b)). Several minor caseins, which were mainly proteolytic fragments of major caseins, showed a weak ability to bind K88ab. Neither β-Casein nor κ-casein appeared to bind K88ab pili.

3. Effect of Carbamylation and D-Galactsoamine

Bovine α_{s1}-casein, human serum IgG, porcine colostral IgG, and pig brush border membrane were carbamylated with cyanate and subjected to dot blot assay (Fig. 2.7). Human serum IgG, porcine colostral IgG, and brush border membrane showed ability to bind K88ab pili, while α_{s1}-casein was somewhat weaker. Carbamylation completely inhibited binding of K88ab pili in all samples examined. Binding by controls (treatment without cyanate) was similar to untreated samples. Addition of D-galactosamine produced a strong inhibition of binding of K88ab pili in all samples. In the absence of K88ab pili, little interaction could be detected between antiserum used in dot blot and all the proteins and brush border membrane.

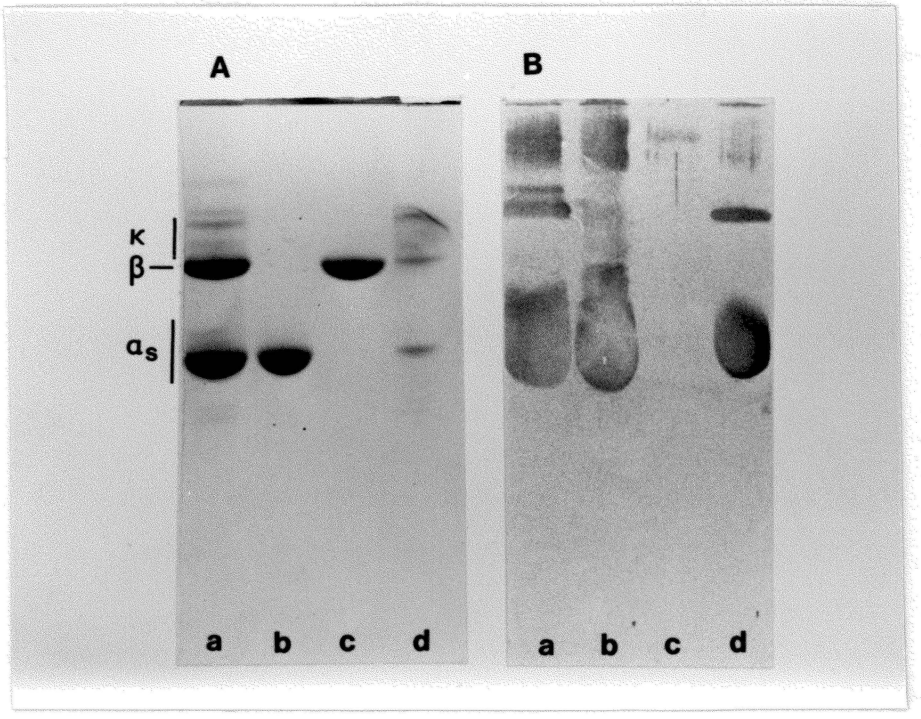

Figure 2.6. Urea gel elctrophoresis (A) and Western blot (B) of cow caseins. (a) whole casein, (b) α_{s1}-casein, (c) β-casein, (d) crude κ-casein. The Greek letters at the left side indicate the locations of α_{s1}-, β-, and κ-casein from cow milk based on the review by Swaisgood (170).

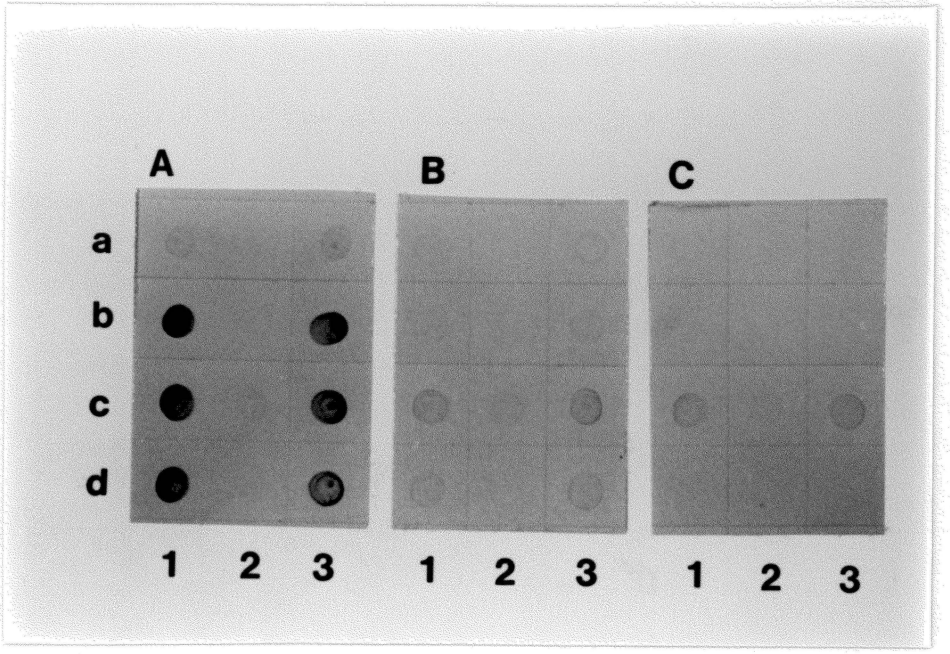

Figure 2.7. Binding of K88ab pili by bovine α_{s1}-casein (a), human serum IgG (b), porcine colostral IgG (c), and brush border membrane (d) in dot blot assay. (A) binding of K88ab; (B) binding of K88ab in the presence of 100 mM D-galactosamine; (C) dot blot without K88ab; (1) no treatment; (2) carbamylation with cyanate; (3) treatment without cyanate.

CHAPTER FIVE

DISCUSSION

Adhesion of K88ab pili to pig brush border membrane has been shown to depend on phenotype. Pigs have been classified as being adhesion-positive and adhesion-negative (155). Pigs have been further classified based on the adhesion patterns of the K88 subtypes, K88ab, K88ac and K88ad (14). This high host specificity suggests the presence of specific complex receptors for each K88 subtype. However, K88-positive E. coli also adhere to brush border membrane and mucus from human (36), mouse (107), and horse (174); milk fat globule membrane from pig and human (142); and erythrocyte membrane from guinea pig, chicken, and pig (164, 134). These reports indicate that receptors for K88 pili are widely distributed in brush border membranes, various plasma membranes and proteins in a variety of animal species.

Although the phenomena of K88 pili adhesion has been extensively studied, the nature of receptor for K88 pili has not yet been determined. Carbohydrate has been suggested to be involved based on decrease in K88 pili adhesion to brush border membrane and mucus following periodate treatment or addition of amino sugars, glycopeptides and glycoproteins. However, attempts to elucidate the carbohydrate structure of K88 pili receptor

has produced contradictory results (65, 153, 8). Some confusion on the nature of receptor sites for K88ab pili may be attributed to absence in the glycoproteins of amino sugars and oligosaccharides inhibitory to binding of K88 (153, 107) as well as undefined structure of glycoproteins used in previous studies (8, 65). The key evidence for involvement of carbohydrate in the receptor for K88 pili is the decrease in binding obtained with periodate-oxidized brush border and mucus. The present study used Western blot to determine the effect of periodate-oxidation and carbamylation on K88ab binding.

The ability of D-galactosamine and other amino sugars to inhibit binding of K88ab pili to brush border membrane in inhibition assay (Table 1.1) and Western blot (Fig. 1.3 and 1.4) was in agreement with results reported in previous studies (153, 107). Similarities in results also would indicate that similar binding mechanisms were involved in both inhibition assay and Western blot. Sellwood (153) reported that compounds possessing free amino groups, as well as amino sugars, inhibit binding of K88 pili by brush border membrane. Inhibition by amino sugars is interesting, since amino sugars which would be positively charged at neutral pH, are not normally present in the oligosaccharide moieties of glycoproteins. However, the free amino groups may be an important part of the receptor

for K88ab pili. Sellwood (153) reported that treatment of brush border membrane with 2.5 % glutaraldehyde drastically decreased binding of K88 pili. He suggested that the decrease resulted from reduction in the mobility of receptors as well as in the fluidity of the membrane through the cross-linking action of glutaraldehyde. He dismissed the possibility that glutaraldehyde might inhibit binding of K88 pili by interaction with ε-amino groups of lysine. Gibbons et al. (65) reported that although intact bovine submaxillary glycoprotein and porcine submaxillary glycoprotein strongly inhibited hemagglutination, neither the polypeptide portions nor the oligosaccharide fractions of these glycoproteins had any effect. Laux et al. (107) suggested that the K88ab-binding site of the receptor contains a component stereochemically similar to D-galactosamine. Thus, current reports in the literature seem to suggest that both carbohydrate and polypeptide are involved in the receptor for K88ab pili.

Laux et al. (107) reported two receptor proteins (M.W. 57,000 and 64,000) for E. coli K-12 (K88ab) in mucus from mouse. Mouse brush border membranes contained the same two receptor proteins as well as an additional receptor protein (M.W. 91,000). The K88ab receptor pattern of pig brush border membrane was more complex than the counterpart from mouse (107). Although the molecular weights of two major

receptor proteins (M.W. 57,000 and 61,500) of pig brush border membrane (Fig. 1. 3) were similar with those of mouse brush border membrane, pig brush border membrane also contained numerous additional minor receptor proteins. The increase in complexity can be attributed to the presence of more receptors for K88-positive E. coli and to the difference in methods used in the two studies. The receptor polypeptides (M.W. 26,500 and 15,500) in isolated mucus (Fig. 1.4) seem to be produced from proteolysis of the receptor polypeptide (M.W. 27,500) in crude mucus during trypsin digestion. However, the receptor polypeptides detected in the pig brush border membrane were not identical with those in mucus. Laux et al. (107) suggested the presence of the same receptor polypeptide in their preparations of mouse brush border membrane and mucus was due to incomplete separation of mucus and brush border by the methods they used.

Sodium metaperiodate reacts with carbohydrates to cleave the linkage between carbons carrying vicinal hydroxyl groups (111). Nonreducing terminal monosaccharides are always oxidized, but the susceptibility of internal monosaccharides depends on glycosidic linkage involved. Concanavalin A recognizes nonreducing terminal α-linked mannose residues and internal α-linked mannose residues which are substituted at position 2 (10, 130).

The carbohydrates normally recognized by concanavalin A are destroyed by periodate oxidation.

In our studies, periodate oxidation decreased, but did not completely destroy, binding of K88ab by receptor polypeptides in the brush border membrane and mucus (Fig. 1.5). However, decreases in the K88ab receptor activity in periodate-oxidized samples corresponded to decrease in the intensity of corresponding polypeptides in polyacrylamide gel electorphoretic patterns (Fig. 1.5). Periodate treatment did result in complete oxidation of nonreducing terminal residues and periodate-oxidized internal residues of oligosaccharide moieties in glycoproteins, since receptor polypeptides in periodate-treated mucus could no longer bind concanavalin A (Fig. 1.5). These results suggest that binding of K88ab pili does not depend on nonreducing terminal residues and periodate-oxidized internal residues of oligosaccharide moieties, but on either periodate-resistant carbohydrates or the nascent polypeptide backbone. These results are in contrast with other studies (65, 107, 153), in which brush border membrane and mucus were treated with periodate. These studies (65, 107, 153) showed that treatment with periodate drastically reduced binding of K88 by brush border membrane and mucus and that periodate-treated glycoproteins do not inhibit hemagglutination. As a result, the authors

suggested that carbohydrates are an important component of the receptor for K88ab pili.

Periodate oxidation of polysaccharides produces compounds which have dialdehydes (73, 149, 111). The dialdehyde derivatives provide a reactive functional group for the attachment of proteins. Under mild conditions, above pH 7, nucleophilic attack by ε-NH_2 groups of lysyl residues in protein can result in formation of carinolamine which results in cross-linking of proteins with oligosaccharides of glycoproteins (149, 94). In the SDS gel electrophoretic patterns of treated brush border membrane (Fig. 1.6 A(c)) and mucus (Fig. 1.6 A(f)), disappearance of polypeptides and appearance of high-molecular-weight polypeptides, which did not penetrate the separating gel, indicate aggregation has occurred as a result of periodate treatment. Decreases in binding of K88ab to the periodate-oxidized brush border membrane and mucus in previous studies (153, 107) may also have occurred as a result of aggregation of brush border membrane. Subsequent decreases in binding may have been due to decreases in the number of receptors accessible to K88 pili or bacterial cells rather than to oxidation of carbohydrate receptors. Gibbons et al. (65) reported that periodate oxidation destroyed the inhibitory activity of human A and H substances and porcine submaxillary glycoprotein on

hemagglutination mediated by K88 pili. However, reduction with sodium borohydride and removal of terminal polyhydric alcohol after periodate oxidation resulted in expression of some hemagglutination activity. Periodate treatment of glycoproteins and membranes should be used carefully to determine dependence of receptor activity on carbohydrate structure.

Pig brush border membrane should be the richest source of oligosaccharide moieties with receptor activity for K88ab pili. Exhaustive digestion with pronase should only affect the polypeptide backbone when producing glycopeptide preparations. The inability of glycopeptide prepared from brush border membrane to inhibit binding of K88ab by brush border membrane (Table 1.2) suggests that the receptor activity resides in the polypeptide backbone rather than in the carbohydrate. However, proteolysis of receptor glycoproteins could convert multiple receptor oligosaccharides in glycoproteins into single oligosaccharide in solution. The oligosaccharides dispersed in solution may no longer possess receptor activity due to low association constant of receptor oligosaccharides and pili (110). These results suggest that the recognition site for K88ab pili in pig brush border membrane and mucus was not a carbohydrate oxidized by periodate but rather the polypeptide backbone or

carbohydrate which is resistant to periodate oxidation and present multivalently in the glycoprotein.

Carbamylation with cyanate chemically modifies amino group present in protein and neutralizes the positive charge. Carbamylation of brush border membrane destroyed binding of K88ab pili by receptor polypeptide in Western blot (Fig. 1.7) and in dot blot (Fig. 2.7). As expected, the treatment did not modify the strucuture of carbohydrate recognized by concanavalin A. These results suggest that amino groups present in brush border membrane polypeptides are the essential parts of the recognition site for K88ab pili. Binding of K88ab pili is the consequence of interaction between pili and protein rather than between pili and carbohydrate. Since carbamylation decreases the overall positive charge of proteins, the inability of K88ab pili to adhere to treated brush border membrane may be due to charge repulsion. However, since the treatment did not decrease binding of concanavalin A, the decreases in K88ab binding do not appear to be due to simple charge repulsion but to modification of amino groups in recognition site of receptor for K88ab pili. The involvement of amino-group in binding of other proteins has also been reported. Both α- and ε-amino groups of protein have been reported to be involved in the reaction of antibody with antigens (24) as well as binding of lipoprotein to cell surface receptors

(177).

Mucus is a slimy, and visco-elastic gel secreted by goblet cells which covers the epithelial surfaces of the small intestine. The molecule responsible for the viscous and gel-forming properties is mucus glycoprotein, which has molecular weights greater than 10^6 and contains high levels of carbohydrate (more than 50 %). Pig small intestinal mucus glycoprotein has a molecular weight of 1.7×10^6 (114). Whether adhesion of bacteria to mucus facilitates colonization of the intestinal tract has not been clearly established (28, 29, 52, 150). Since mucus forms a gel matrix which is impermeable to large molecules (52) and is continuously released into the intestinal lumen by goblet cells, bacteria which adhere to epithelial cells should be able to grow in and quickly penetrate through mucus gel. In the present study, mucus isolated from the intestines of 2-day-old piglets had more receptor polypeptides for K88ab pili than mucus prepared from older pigs (Fig 1.9). These results suggest that adhesion of K88-positive ETEC to mucus may aid colonization and explain why new-born piglets are more susceptible than older piglets to diarrhea mediated by K88-positive ETEC.

Polypeptide patterns obtained for small intestinal mucus prepared from pigs of various ages were distinctively different from each other. However, the polypeptide and

receptor patterns obtained for brush border membranes from pigs of various age were more similar (Fig. 1.8). Mucus seemed to undergo more structural and biochemical changes than brush border membrane during growth and development of pigs. Brush border membrane appeared to already possess a certain structural integrity before birth of the piglet, while mucus in new-born piglets may be biochemically and physically immature in protecting the intestinal wall against bacterial colonization.

Colostrum and milk from sows immunized with K88-positive ETEC possess anti-adhesivie activity *in vitro* (127, 145, 146). Sows susceptible to diarrhea by K88-positive ETEC secret colostrum and milk which contain more anti-adhesive activity than resistant sows (154). The anti-adhesive activity has been attributed to the anti-K88 antibody in colostrum and milk (154, 127, 91). In addition to anti-K88ab antibody, milk and colostrum contain substances which may have structures similar to receptors for K88 pili located in brush border membrane. The receptor-analogue substances in mammary secretion may compete with the true receptors for K88 in brush border membrane and inhibit the adhesion of K88-positive ETEC to intestinal mucosa (142). Previous studies have reported that low molecular weight glycopeptide in colostrum (65) and MFGM in milk (141) inhibit hemagglutination of guinea

pig erythrocytes by K88-positive E. coli. K88-positive E. coli were also shown to attach to MFGM from sow milk (142).

In the present study, a binding assay incorporating both K88 pili and intestinal brush border membrane instead of hemagglutination was used to identify fractions of milk and colostrum which inhibit binding of K88ab pili. Additionally, Western blot assay was used to identify the proteins which bind K88ab pili. All fractions from sow milk and colostrum with significant inhibition contained at least one protein which bound K88ab pili. Casein was the only fraction from cow milk which significantly inhibited binding of K88ab pili. α_{s1}-Casein was identified as the component responsible for bindig K88ab pili. Whey and MFGM from cow milk contain few proteins which significantly bind K88ab (results not shown). The agreement between results obtained from inhibition assay and Western blot indicate that competition between receptors in the brush border membrane and proteins in milk and colostrum is responsible for inhibition of K88ab binding by brush border membrane.

MFGM from sow milk and colostrum significantly inhibited binding of K88ab to brush border membrane, but MFGM from cow milk did not. However MFGM from cow milk was able to bind K88ab pili, although the level of binding was much less than obtained with sow MFGM. These results were in partial agreement with Reiter (142) who showed

attachment of K88-positive E. coli strains to MFGM from sow milk but not from bovine milk. The molecular weights of the major MFGM polypeptides in sow milk (M.W. 155,000 and 68,000) which bind K88ab (Fig. 2.2) indicate that they are xanthine oxidase and butyrophilin, respectively (76).

Some polypeptides present in colostral whey fractions were identified by a combination of SDS-electrophoresis and double immunodiffusion. The two reduced polypeptides (M.W. 57,000 and 26,000) (Fig. 2.4) and the unreduced polypeptides (M.W. 170,000) (Fig. 2.5) appeared to be immunoglobulin heavy chain, light chain, and whole IgG, respectively. The other unreduced polypeptides with higher molecular weight in fraction A were IgA and IgM. The results demonstrate that light chain, heavy chain, and immunoglobulin bind K88ab (Fig. 2.4) with whole immunoglobulins binding more strongly than reduced chains (Fig. 2.5). Inhibition of K88 binding by whey was not due to the presence of anti-K88ab antibody but to binding by immunoglobulin protein. This conclusion was based on 1) the relatively weak inhibition of K88ab binding provided by sow whey (Table 2.1), 2) the absence of detectable anti-K88ab antibody in double immunodiffusion assay, and 3) D-galactosmine inhibition of K88ab binding by porcine colostral IgG in dot blot assay (Fig. 2.7). Sellwood (154) reported that colostrums from sows resistant to diarrhea

caused by K88-positive ETEC have some inhibitory activity, although levels were much less than from colostrum of susceptible sows. The ability of immunoglobulin chains to bind K88ab (shown in Western blot (Fig 2.4 and 2.5) and dot blot (Fig. 2.7)) may correspond to inhibition by colostral whey which is secreted by sows resistant to diarrhea and in which little anti-K88ab antibody is present. The low level of inhibition detected in cow whey may be due to the small amount of immunoglobulins in cow milk, since the major proteins in cow whey are β-lactoglobulin and α-lactalbumin (46).

The only major component of bovine casein which binds K88 pili was α_{s1}-casein (Fig. 2.3 and 2.6). Additionally low levels of binding were detected in the region of the gel in which proteolytic fragments of the caseins usually migrate (Fig. 2.6). Neither β- or κ- casein appeared to have ability to bind K88ab pili. In previous studies, K88-positive E. coli appeared to be bound by glycoconjugates with carbohydrate as an essential recognition site (65, 8, 107, 153). However, results obtained in this study indicate that proteins, such as α_{s1}-casein and the light chain of immunoglobulin, also bind K88ab pili. Dot blot showed that carbamylation completely inhibited binding of K88ab pili by bovine α_{s1}-casein, human serum IgG, porcine colostral IgG, and brush border

membrane. These results suggest that amino groups present on these proteins, as well as the polypeptides in brush border membrane, are important to binding of K88ab pili. The interaction of K88ab pili with proteins in milk and colostrum should involve mechanisms similar to binding of K88ab pili by brush border membrane. The ε-amino group of lysine in protein should be particularly important in binding of K88ab pili. The positive charge associated with the binding site for K88ab pili is also important. However, we do not know at this time what other protein structures may be required to bind K88ab pili with high affinity.

The inhibition of binding of K88ab pili may also occur in the small intestine of pigs infected with ETEC. When allowed to suckle sow colostrum and milk, piglets should obtain large amounts of receptor analog substances for K88ab pili as well as antibodies. The strong binding of K88ab by cow α_{s1}-casein is interesting. Strains of ETEC containing K88 do not produce diarrhea in calves. α_{s1}-Casein may partly contribute to the absence of diarrhea in calves caused by K88-positive E. coli. Further identification of mechanisms involved in binding of K88 may lead to the addition of binding inhibitors to the the feed of piglets to aid in diarrhea reduction.

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BINDING MECHANISM OF K88AB PILI PRODUCED BY ENTEROTOXIGENIC *Escherichia coli*

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(ABSTRACT)

Binding of K88ab pili by brush border membrane and mucus from pig small intestine was characterized by inhibition assay and Western blot. In Western blot, K88ab pili were bound by two major brush border membrane polypeptides with molecular weight of 61,500 and 57,000 in addition to numerous minor polypeptides and a major mucus polypeptide with molecular weight of 27,500. The results from Western blot assays with periodate oxidized and carbamylated brush border membrane and inhibition assay with brush border membrane glycopeptide suggest that amino groups (rather than carbohydrate) present on the protein moiety are a part of the recognition site for K88ab pili of receptor polypeptides in brush border membrane. Differences were obtained in the binding patterns of K88ab pili when brush border membranes were prepared from small intestines obtained from 2-, 21-, and 42-day-old piglets as well as adult hogs. Binding of K88ab pili by mucus polypeptides was greater when prepared from small intestines obtained from 2-day-old piglets than from piglets of other ages and adult

hogs.

In inhibition assay, most fractions from sow milk and colostrum inhibited binding of K88ab pili. After gel filtration of colostral whey, fractions which contained IgG, IgA, and IgM produced the strongest inhibition of K88ab binding. Among fractions prepared from cow milk, casein and skim milk significantly inhibited binding of K88ab pili. In Western blot, α_{s1}-casein, immunoglobulin chains, and MFGM polypeptides in sow milk and colostrum were shown to be able to bind K88ab pili. Additionally, α_{s1}-casein was the major protein in bovine milk responsible for binding K88ab pili. In dot blot assay, IgG as well as brush border membrane could strongly bind K88ab pili. However, bovine α_{s1}-casein showed only weak binding of K88ab pili. Binding of K88ab pili to these proteins and brush border membrane was inhibited by carbamylation and by addition of 100 mM D-galactosamine. The results suggest that the K88ab-binding proteins in milk and colostrum compete to bind K88ab pili with the receptors in the brush border membrane and that mechanisms involved in binding of K88ab pili by these proteins is similar to that by brush border membrane.